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The ability of certain human breast cancer cells (HBC) to metastasize to bone may be related to cellular expression of the small acidic sialoproteins osteopontin (OPN) and bone sialoprotein (BSP). Expression of these proteins may promote primary tumor cell invasion, and/or the development of bone metastases. The purpose of this study is to compare BSP and OPN expression in HBC to bone metastatic potential and to examine potential breast cancer cell responses to BSP. Our results indicate expression of BSP and OPN in a number of primary breast tumors and their corresponding bone metastases, and expression of OPN in both the LCC-15MB and MDA-MB-435, two invasive and metastatic HBC cell lines. Interestingly, BSP expression in HBC cells appears to be absent. We have also employed purified rat BSP, human recombinant BSP fragments and novel BSP-derived RGD peptides to show that BSP confers positive migratory, proliferative and adhesive effects upon the MDA-MB-231 HBC cell line *in vitro,* and that these effects are modulated through specific integrins. This proposal addresses research which will be potentially important in the diagnosis and, ultimately, treatment of breast cancer metastasis to bone.

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III. Introduction

Bone is the second most common organ in which breast cancer metastases develop, being found at autopsy in over 80% of patients dying from carcinoma of the breast (1). Osteolysis is often associated with breast-bone metastasis, resulting in significant pain and reduced mobility as well as other clinical diseases such as hypercalcemia, spinal cord compression and pathological fractures (2). Although bone metastasis is considered a selective process, the molecular mechanisms responsible for preferential targeting of human breast cancer (HBC) cells to bone are not well understood. Systemic as well as local factors appear to be involved. Since the bone marrow is highly vascularized, it not only has a higher exposure to tumor cells, but also to various systemic hormones, cytokines and growth factors which may promote growth of metastatic cells (3). The organic phase of bone also contains several of these substances including transforming growth factor-beta, insulin-like growth factor, basic fibroblast growth factor and platelet derived growth factor, which have been shown to be mitogenic for many cell types including carcinoma cell lines (4). In addition, bone contains several cytokines including interleukins 1 and 6 which may themselves induce cell proliferation, but can also up-regulate production of matrix-degrading components such as collagenases and urokinase type plasminogen activator, resulting in increased tumor cell invasiveness (3). The stimulation of these proteases can then facilitate osteolysis by increasing osteoclastic resorption. Finally, inherent production of proteolytic enzymes, expression or loss of cell adhesion molecules, migratory capability, and growth responsiveness to bone-localized growth factors and hormones are all specific properties of tumor cells which may aid in metastasis to bone. The development of effective methods to quantitate bone metastasis in current *in vivo* models as well as studying the *in vitro* characteristics of bone metastasis-derived cell lines will further elucidate mechanisms of tumor metastasis to bone.

Recent reports in the literature have demonstrated expression of the small acidic sialoproteins osteopontin (OPN) and bone sialoprotein (BSP) in primary breast lesions, and suggested that they may be involved in mammary microcalcifications and the ability of certain breast cancer cells to metastasize to bone (5-7). Additionally, these studies proposed that increased expression levels of

BSP and/or OPN may indicate a higher likelihood for lymph node metastasis. Although both of the bone matrix proteins were shown to be present in paraffin-embedded primary breast tumors, *in situ* hybridization studies have also shown macrophages to be a source of tumor-associated OPN (8,9).

BSP and OPN are acidic, sulfated glycoproteins which are primarily secreted by osteoblasts and osteoclasts, and thought to take part in bone formation by binding to hydroxyapatite (10,11). Recent work has also indicated that BSP stimulates proliferation of mouse preosteoblast cells (12). In normal bone, BSP is one of the most abundant non-collagenous proteins, and is usually associated with newly synthesized osteoid, lending support to a role in bone mineralization (13). Both BSP and OPN are post-translationally modified and while BSP has repeated clusters of glutamine, OPN instead, contains aspartic acid stretches; these are thought to allow binding of hydroxyapatite crystals in the bone mineralization process (14,15). The complete nucleic acid sequences of human, rat, mouse, and cow BSP and OPN, have been determined encoding an approximately 80-kD, highly modified protein with a conserved integrin-binding RGD (Arg-Gly-Asp) tripeptide and three polyglutamic acid domains which have hydroxyapatite-binding abilities. Rat and human BSP are the most highly conserved, having a 70% sequence homology, including the RGD domain which is necessary for cell-binding abilities of BSP as well as several other extracellular matrix proteins including vitronectin and fibronectin (15-19). Additionally, the few amino acids directly flanking the RGD domain are almost identical in rat and human BSP. This RGD domain of BSP has been shown to mediate the attachment of osteosarcoma cells and osteoclasts *in vitro* (20-22) and may also be involved in the interaction between BSP and collagen I (23). The RGD domain of osteopontin (OPN) has been implicated in osteoclast-mediated bone resorption, initiation of cellular signaling pathways and increased adhesion and migration of HBC cells (22,24-26). Aside from its RGD-mediated activities, OPN also appears to have non-RGD domains which promote cell attachment (27), and a similar, non-RGD-related cell adhesion mechanism has been evidenced for BSP (28,29). Recently, the MDA-MB-231 human breast cancer cell line has been shown to recognize synthetic peptides encompassing the RGD sequence of human BSP (30). In addition, these peptides were able to inhibit tumor cell adhesion to bone matrix, suggesting that BSP may support adhesion of human

breast cancer cells to the bone through RGD-binding integrins. Furthermore, an integrin-mediated interaction between bone sialoprotein and tumor cells may also promote metastasis by inducing increased cellular proliferation and/or migration.

The integrins, a large family of α/β heterodimeric transmembrane proteins, mediate cell-cell and cell-substratum adhesion and interact with many extracellular matrix components including fibronectin, vitronectin, collagens and laminin. Integrins are often expressed in high numbers on the cell surface and depending on cell type, can sometimes bind multiple ligands or show alternative specificity to influence adhesion, migration or proliferation in response to the same ligand (31). Recent work has also demonstrated a role for integrins in initiation of signaling pathways due to integrin activation or ligand-binding (32). At this time, there is an abundance of research detailing changes in expression or function of integrins which occur upon malignant transformation, and which may help to explain their undifferentiated phenotype and uncontrolled growth rates. For example, in mammary adenocarcinomas, there is evidence for decreased expression of the $\alpha 2\beta 1$ and $\alpha 5\beta 1$ integrins (33,34) and $\alpha v\beta 3$ was shown to be involved in increased adhesion, motility and metastasis of a large cell lymphoma and in the growth of human melanoma cells (35,36) A requirement for $\alpha v\beta 3$ in angiogenesis associated with wound healing and tumorigenesis was also recently demonstrated (37,38).

Because BSP contains an RGD domain, it is capable of supporting integrin-mediated attachment of certain cells in normal bone. Several integrins including $\alpha v\beta 3$ and $\alpha 5\beta 1$ have been implicated in both attachment of osteoclasts to bone matrix and transmittance of bone matrix-derived signals across the osteoclast plasma membrane (22,39,40). On the other hand, bone matrix was also demonstrated to mediate adhesion of human prostate carcinoma via the $\alpha 2\beta 1$ integrin (41) and breast carcinoma cells via the $\alpha v\beta 3$ integrin (42). Because malignant as well as normal cells use cell surface integrins to interact with the bone matrix, BSP may influence HBC cell metastasis to bone by imparting upon the tumor cells an increased capacity to adhere to, proliferate in, and migrate through the bone matrix. In addition, since BSP has been shown to be responsible for stimulating bone resorption (43), integrin-mediated attachment to bone matrix may also provide a direct

mechanism for tumor cell osteolysis of bone. Such BSP-mediated interactions between HBC cells and bone matrix may contribute to the development of bone metastasis.

At this time, it is not known how BSP may influence metastasis, and what percentage of bone-metastasizing breast cancer cells express BSP and/or OPN. It is possible that the invasion process simply requires the ability of a tumor cell to respond to these bone matrix proteins (paracrine mechanism), while inherent expression (autocrine mechanism) results in a more selective metastasis to bone. Also, the bone environment may induce cancer cells to express certain bone matrix proteins to further facilitate metastatic colonization and stimulation of osteolysis. Identifying some these mechanisms will contribute to a better understanding of the metastatic pathway, and allow us to eventually prevent the spread of malignant cells to bone. The purpose of this study is to compare BSP and OPN expression with metastatic potential and to examine potential responses of breast cancer cell to BSP and OPN.

IV. Body

A. Experimental Methods, Assumptions and Procedures

Cell culture. The isolation of LCC15-MB was recently described (44), and the other established human breast cancer (HBC) cell lines used in comparison (T47D, MCF-7_{ADR}, SKBr3, MDA-MB-453, BT549, 7MDA-MB-436, MDA-MB-231, MDA-MB-435, Hs578T) were originally obtained from the ATCC (Rockville, MD). MCF-7 cells, originally obtained from Dr. Marvin Rich (Michigan Cancer Foundation), were provided by the Lombardi Cancer Center Cell Culture Resource and the UMR-106-01BSP rat osteosarcoma cell line was obtained from Dr. Larry Fisher (NIDR, NIH, Bethesda, MD). All cell lines were grown in Richter's Improved Minimal Essential Medium (IMEM, Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD) and maintained at 37°C in 5% CO_2, 95% air. Cultures were confirmed as Mycoplasma free by the Lombardi Cancer Center Cell Culture Shared Resource using the Genprobe kit (Gen-Probe, San Diego, CA).

BSP Reagents.

BSP, recombinant fragments and cyclic RGD peptides: Rat bone sialoprotein (BSP) was isolated from the UMR-106-01BSP rat osteosarcoma cell line as previously described (45,46), the recombinant BSP fragments synthesized in *E. coli* (amino acids 258-317; designated BRB 9 and 10) (47), and the cyclic and linear BSP-derived RGD peptides, CBA4 and BA3 (EPRGDNYR; denoted CNB and CP3 in reference 30), respectively, were synthesized and kindly provided by Dr. Frank Robey (NIDR, NIH, Bethesda, MD). Figure 1 illustrates the different BSP preparations which we used in this study, highlighting the RGD consensus sequence as a reference point in each form. Note that the recombinant fragments BRB 9 and 10 are identical with the exception of a mutation of the RGD to KAE in BRB 10.

Antibodies: The integrin blocking antibodies against αv (MAb L230), αvβ3 (LM609, MAb 1976) and αvβ5 (MAb clone P1F6, mouse ascites) were obtained from ATCC (Rockville, MD), Chemicon International (Temecula, CA) and Gibco BRL (Gaithersburg, MD) respectively, and the

FITC-conjugated goat anti-mouse IgG was also purchased from Gibco BRL. The vitronectin used in the adhesion assay was a generous gift from Dr. Steven Akiyama (NIDR, NIH, Bethesda, MD), and the Alamar Blue vital dye was from Biosource International (Camarillo, CA).

Immunocytochemistry. Archival paraffin blocks were obtained for the primary breast tumor and bone metastasis with the help of Dr. Norio Azumi (Department of Pathology, Georgetown University Medical Center). Material from the nude mouse subcutaneous xenografts were fixed in formalin and embedded in paraffin following routine procedure. Sectioning of the paraffin blocks onto *pro-bond+* slides (Fisher Scientific, Pittsburgh, PA) was performed by the Lombardi Cancer Center Tissue Shared Resource, and immunohistochemistry of paraffin sections was performed with the autoimmunostainers (TechMate 1000, BioTek Solutions, Santa Barbara, CA and Ventana 320, Ventana Medical System, Tuscon, AZ) using a standard peroxidase and avidin-biotin-complex method with diaminobenzadine as the chromogen. All the reagents except for the primary antibodies were supplied by the autoimmunostainer manufacturers. We used rabbit polyclonal antibodies to BSP and OPN (LF83, 1:500 dilution and LF19, 1:200 dilution, respectively; gifts from Dr. Larry Fisher, NIDR, NIH) in conjunction with microwave antigen retrieval. Paraffin-embedded plugs of each cell line were prepared by harvesting cells at approximately 80% confluence from 100 mm^3 tissue culture plates, washing with 1X Phosphate buffered saline (PBS, Gibco BRL, Gaithersburg, MD) and immediately adding a 10% formalin solution to fix the cells. The plugs were then processed routinely and stained for BSP or OPN as described above.

Northern Analysis. Cytoplasmic RNA was prepared by standard methods using 4M guanidinium isothiocyanate (Gibco BRL, Gaithersburg, MD) from cultured HBC cell lines (48). Approximately 20 µg of mRNA was loaded onto a 1% agarose gel containing 10% formaldehyde and transferred to a nylon membrane (Hybond-N, Amersham Life Sciences, Arlington Heights, IL). cDNA probes for BSP (B65G) and OPN (OP10) (16,28,49,50) were kindly provided by Dr. Fisher, NIDR, and radioactively labeled with ^{32}P using random hexamer priming (Boerhinger Mannheim, West Germany).

Cell migration assay. Migration experiments were performed in triplicate essentially as previously described (51) but adapted for the 48-well Boyden chamber apparatus (Neuroprobe, Cabin John, MD). EHS tumor-derived type IV collagen in 0.5M sodium acetate (50 μg/filter), a gift from Dr. Hynda Kleinman (NIDR, NIH), was applied to polycarbonate filters (12.0 μm pores, PVP-free, Costar, Cambridge, MA) and allowed to dry. Full length rat BSP (1 μM), recombinant BSP fragments or RGD peptides (1 and 10 μM, respectively) were reconstituted in phosphate buffered saline (PBS, Gibco BRL, Gaithersburg, MD), diluted in IMEM supplemented with 0.1% bovine serum albumin (BSA, Sigma, St. Louis, MO), and used as the chemoattractant in the bottom chamber. IMEM plus 0.1% BSA (IMEM-BSA) and IMEM with 10% FBS (IMEM-FBS) were used as baseline and positive controls, respectively. Cells were harvested with trypsin (Gibco BRL, Gaithersburg, MD), washed twice with serum-free IMEM, resuspended in IMEM-BSA, and added to the top chamber at a density of 15,000 cells/well. Chambers were incubated in a humidified incubator at 37°C in 5% CO_2 for 18 hours. The cells which had traversed the filter and spread on its lower surface were stained with Diff-Quik (American Scientific Products, McGaw Park, IL), and quantitated by counting the number of cells per 9 representative fields at a magnification of 20X. Data presented are an average (with standard deviation) number of cells per nine representative microscopic fields.

Cell proliferation assay. Proliferative responses (anchorage-dependent) were assessed in triplicate in 96-well plates with full length BSP (1 μM), recombinant BSP fragments (1 μM) and RGD peptides (10 μM) diluted in IMEM-BSA to the concentrations indicated. Similar to the migration assay, IMEM-BSA and IMEM-FBS were used as baseline and positive controls, respectively. Cells were harvested with trypsin and plated in IMEM-FBS at a density of 1500 cells/well at 37°C, 5% CO_2. On the following day, the media was removed, the cells washed twice with serum-free IMEM, and the experimental media (proliferative agent in IMEM-BSA) added. Cell number was assayed after 1, 3 and 5 days using Alamar Blue (Biosource International, Camarillo, CA), a non-toxic dye which living cells can reduce from a blue to red color. After incubation with the dye for 24 hours, extent of proliferation was measured by absorbance at 570 and 600 nm, determined using a 96-well plate ELISA reader (Dynatech MR700, Dynatech Laboratories, Chantilly, VA).

Flow cytometric analysis. 50,000 cells were harvested with trypsin in the log phase of growth, washed with PBS and resuspended in 100 µl 3% BSA/IMEM with the anti-integrin antibodies αvβ3 (0.2 µg) and αvβ5 (1:500 dilution), followed by incubation for 1 hour on ice. After washing twice with 1 ml IMEM/3% BSA, 100 µl FITC-conjugated goat anti-mouse IgG in IMEM/3% BSA was added to the cells, and incubated for 1 hour on ice. Finally, cells were again washed with IMEM/3% BSA and analyzed for surface integrin expression by flow cytometry (FACSplus, Becton Dickinson, San Diego, CA). For cell sorts, single cells enriched for either αvβ3 or αvβ5 (top 10%) were selected, cloned into 96 well plates and grown for 2-3 weeks through a single passage, after which they were subjected to a second negative single cell sort to exclude remaining cells which retained expression of the other integrin. Although populations were relatively enriched for one particular integrin, there was continued low expression of the other integrin. Sorted cells were used within 2 weeks of the second sort and selective integrin expression profile was relatively stable for one month.

Attachment assay. Attachment assays were performed essentially as previously described (52) in triplicate in 96-well plates pre-coated with either vitronectin (1 µg/ml), BSP fragments (1 µM) or BSP-derived RGD peptides (10 µM) in PBS for 1 hour at 37°C. The coating solution was then replaced with 50 µl of 3% BSA/PBS added to each well for 30 minutes, 37°C, to block non-specific binding sites. The wells were then washed three times with PBS. Meanwhile, the cells were harvested with trypsin and $2.5x10^5$ cells/ml were washed and resuspended in IMEM/BSA. The cell suspension was incubated with or without integrin blocking antibodies for 1 hour at 37°C before being added (total volume = 100 µl) to each precoated well and incubated for 1 hour to allow for attachment. The supernatant containing the unattached cells was then removed and the attached cells rinsed gently with PBS and stained with 50 µl of 0.05% crystal violet in 25% methanol for 5 minutes. The plate was rinsed three times by immersion in water and allowed to dry at room temperature. The incorporated dye was dissolved in 100 µl 0.1M sodium citrate in 50% ethanol and measured by reading absorbance at 540 nm with a 96-well plate ELISA reader.

In Vivo **Subcutaneous Xenografting in Nude Mice.** Mice were housed in sterile laminar flow rooms at 25°C and 50% humidity. Subcutaneous (S/c) xenografting was performed as previously described for other HBC cell lines (50,51). HBC cells were harvested from near confluence with trypsin, resuspended in PBS at 1 x 10^7 cells/ml, and inoculated bilaterally (5 x 10^6 cells/site) into the mammary fat pad area of 6-8 week old female NCr *nu/nu* nude mice (NCI, Frederick, MD). Mice were monitored daily, and tumor measurements were taken twice weekly for 30 days. At the time of harvest, samples of all organs were fixed in formalin, embedded in paraffin, and analyzed by routine histology (hematoxylin and eosin, H&E).

B. *Results and Discussion*

1. Expression of bone sialoprotein and osteopontin in human breast cancer

Several lines of evidence indicate a distinct specificity to the process of bone metastasis, perhaps the most striking of which is the preference of the bone site for cells of breast and prostate origin (1,3). Reports in the literature have suggested that the ability of certain breast cancer cells to metastasize to bone may be related to cellular expression of the small acidic sialoproteins osteopontin (OPN) and bone sialoprotein (BSP) (7). They are both normally found in bone matrix, but are expressed in approximately 80% of primary mammary carcinomas and associated with microcalcifications in the breast. It is possible that cancer cells secreting OPN and BSP may have an increased affinity for bone and/or may be involved in the bone resorption usually associated with bone metastasis. Therefore, we looked for BSP and OPN expression in a number of primary breast cancers and their associated bone metastases to explore whether a relationship exists between BSP expression in the primary tumor and subsequent bone metastasis.

A preliminary immunohistochemical survey of several randomly selected paraffin-embedded primary breast lesions confirms positive BSP and OPN (Figure 2A-C; OPN data not available) expression in these tumors, and in addition, further studies revealed positive BSP staining in both the primary breast tumor and its associated bone metastasis (Figures 2C and 2D), correlating expression of BSP in the primary lesion with bone metastasis *in vivo*.

We also examined BSP and OPN expression in a variety of human breast cancer (HBC) cell lines ranging from the traditionally non-invasive to the more aggressive lines. The UMR-106-01BSP (UMR) rat osteosarcoma cell line, expressing endogenous levels of BSP, was used as a positive control in the following experiments. Initial experiments which examined RNA levels in these cell lines (by Northern analysis) revealed a complete absence of BSP message in all HBC cell lines assayed (Figure 3A). Interestingly, parallel studies (Figure 3A) revealed OPN RNA expression in both the bone metastatic LCC15-MB cell line and the highly metastatic MDA-MB-435 cells. The

UMR rat osteosarcoma cell line expressed both BSP and OPN, and sample loading was controlled using a probe to the GAPDH housekeeping gene. Corresponding immunocytochemical studies of several of the HBC cell lines similarly demonstrated a lack of BSP staining in all but the UMR cells (Figure 4A-D) while the LCC15-MB and MDA-MB-435 cell lines both stained positively for OPN (Figure 5A-D).

In order to explore whether expression of these proteins might be regulated differently *in vivo*, we selected the MDA-MB-231, MDA-MB-435 and LCC-15 MB HBC cell lines along with the UMR cells for subcutaneous injection into the mammary fat pads of nude mice. Each of these cell lines was tumorigenic and formed tumor xenografts in the animals after a latency period of about 1 week. The tumors were harvested after approximately 1-2 months and processed for RNA by Northern blotting. Similar to the cell line analyses, BSP was not expressed in the nude mouse xenografts , although the MDA-MB-435 and LCC15-MB cell xenografts expressed OPN message (Figure 3B). As expected, the UMR xenograft expressed both BSP and OPN. Finally, results from immunocytochemical studies of the xenografts were similar to those seen at the RNA level, with expression of BSP restricted to the UMR cell xenograft and OPN expression present in the UMR, LCC15-MB and MDA-MB-435 xenografts (Figures 4E-H and 5E-H).

These initial studies confirm that BSP and OPN are expressed in primary mammary tumor specimens as well as their corresponding bone metastases as assayed by immunocytochemistry. Surprisingly, however, none of the established HBC cell lines examined expressed BSP at either the RNA or protein levels. To exclude the possibility that BSP was down-regulated in culture conditions, we grew several cell lines as nude mouse xenografts *in vivo*, but similarly found that they did not express BSP. It is possible that while these tumor cells themselves do not make BSP, other closely-associated cells in the tumor environment are secreting the large quantities of sialoprotein, which might then be adsorbed to the tumor cells. In fact, two previous studies exploring the expression of OPN in paraffin-embedded primary tumor samples found OPN expression in a variety of primary tumor types, by immunocytochemistry, while *in situ* hybridization experiments later showed tumor-associated macrophages to be a major source of OPN in the tumor samples surveyed (8,9). Our

HBC cell studies also show OPN to be expressed in primary breast tumor samples, however, we found that it was expressed by two HBC cell lines, LCC15-MB and MDA-MB-435. This is potentially interesting because both cell lines are very invasive and metastatic, and the LCC15-MB cells are derived from a breast-bone metastasis. In fact, OPN was more directly identified as a metastasis-related gene product by subtractive hybridization in a recent rodent mammary tumor model (53). Ongoing studies are exploring whether the expression of OPN promotes overall metastasis, or, more specifically, bone metastatic potential of certain HBC cell lines. Since no known HBC cell lines express endogenous BSP, we are currently attempting to express BSP exogenously in an HBC cell line, and hope to use this cell line as a model with which to study the effects of BSP production on breast cancer invasion and metastasis, both *in vitro* and in *vivo*. Although it is possible that BSP is not produced by HBC cells directly, these cells would still have the potential to respond to BSP, both in the bone environment and at the site of the primary tumor. Therefore, we have also examined *in vitro* HBC cell responses to BSP, and explored possible domain(s) of the protein which may be responsible for eliciting these responses.

2. Human breast cancer cell responses to bone sialoprotein.

BSP possesses an integrin-binding RGD (Arg-Gly-Asp) domain, which has been shown to mediate the attachment of osteosarcoma cells and osteoclasts *in vitro* (15,21,22). Similarly, the RGD domain of osteopontin was implicated in interactions between bone matrix and osteoclast-mediated bone resorption, initiation of cellular signaling pathways and increased adhesion and migration of HBC cells (24-26). Recently, the MDA-MB-231 human breast cancer cell line was shown to recognize synthetic peptides encompassing the RGD sequence of human BSP (30). In addition, these peptides inhibited tumor cell adhesion to bone matrix, suggesting that BSP may support adhesion of human breast cancer cells to the bone through RGD-binding integrins. Because malignant as well as normal cells use integrins to interact with the bone matrix, we hypothesized that BSP may support HBC cell metastasis to bone by imparting upon the tumor cells an increased capacity to adhere to, proliferate in, and migrate through the bone matrix. These cellular responses are all components of the metastatic cascade, a series of steps through which primary tumor cells

must progress in order to establish at a site of secondary growth (54). We tested our hypothesis by examining responses of the MDA-MB-231 HBC cell line (which does not express BSP or OPN) to purified BSP, recombinant BSP fragments, and RGD-derived BSP peptides (Figure 1). We also used specific integrin blocking antibodies to more specifically elucidate the BSP-integrin interactions which may promote HBC cell metastasis to bone.

MDA-MB-231 responses to BSP and BSP fragments. We first monitored cell migration, proliferation and attachment responses to rat BSP as well as to the recombinant human BSP fragments. As shown in Figure 6A, MDA-MB-231 (MDA-231) cells migrated readily across the filter towards BSP and both BRB 9 and 10. BRB 10 (with a mutated RGD domain) evokes only a slightly smaller migratory response than BRB 9, suggesting that BSP confers both RGD and non-RGD-mediated stimulation of cell migration. Because of our collaborators are specifically interested in the non-RGD component, we have concentrated our efforts on the RGD-mediated aspect of migration.

MDA-231 proliferative responses to rat BSP were also positive, showing an approximately 40% stimulation over basal proliferation in 0.1% bovine serum albumin (Figure 6B). However, the BSP RGD consensus appears to be more important for MDA-231 proliferation than migration, since BRB 10 was much less effective than BRB 9 in increasing proliferation. These effects were seen after 5 days but were not evident at day 3. Purified BSP was only tested at day 4 due to its limited availability.

Using BSA-blocked plastic and vitronectin as negative and positive controls, respectively, we further examined RGD involvement in attachment of MDA-231 cells to BSP (Figure 6C). The cellular adhesion profile mirrored proliferation, but with more pronounced HBC cell attachment to BRB 9 than BRB 10. Cell attachment toward vitronectin is shown as a point of reference. These results suggest that the RGD domain of BSP is required for proliferation and adhesion to BSP, while both RGD and non-RGD motifs are involved in MDA-231 cell migration toward BSP.

MDA-MB-231 responses to RGD peptides. To further characterize the RGD-mediated responses, we examined MDA-231 responses to specific BSP-derived RGD peptides. The cyclic (CBA4) and linear peptides (BA3) contain the RGD consensus sequence flanked on either side by 2 and 3 BSP amino acids, respectively, and have recently been shown to inhibit MDA-231 cell adhesion to bone sections (30). Figure 7A demonstrates that these peptides selectively stimulate cell migration, the cyclic CBA4 peptide being almost twice as potent as the linear BA3 form. In contrast, no effect was seen with the linear fibronectin-derived GRGDSP peptide, suggesting that the BSP-specific flanking sequences are important for eliciting MDA-231 migration.

MDA-231 proliferation (Figure 7B) and attachment (Figure 7C) experiments also showed direct BSP-RGD responses, and again, the cyclic BSP-derived RGD peptide was more active than the linear form in both cases. As with migration, neither the fibronectin-derived GRGDSP peptide nor the GRGESP control peptide supported cellular proliferation or adhesion, indicating the BSP-RGD specificity of these responses. The increased effectiveness of the cyclic form of the BSP-RGD peptide (CBA4) as compared to its linear form (BA3) implies that the tertiary conformation of the BSP molecule is important in conferring migratory, proliferative and adhesive responses, as was indicated previously (30). Interestingly, currently ongoing "checkerboard" Boyden invasion analysis indicates that BSP-RGD-induced migration of MDA-231 cells is due predominantly to chemokinesis rather that chemomigration, suggesting that BSP may have an overall inductive effect on tumor cell motility.

Effects of integrin blocking antibodies on cellular responses. The observation of novel RGD-mediated BSP responses in MDA-231 cells raised the question of which cell surface integrins might be responsible for these effects. Because both OPN and BSP have been shown to interact with αv-containing integrins, we first tested an anti-αv antibody. As seen in Figures 8A-8C, the L230 antibody decreased migration, proliferation and adhesion of the HBC cells to CBA4 by approximately one half, and to BA3 by a lesser extent. Further delineation using blocking antibodies to either $\alpha v\beta 3$ or $\alpha v\beta 5$ integrin demonstrated that the cell response was not inhibited by the $\alpha v\beta 5$ blocker, but proliferative and adhesive responses were significantly decreased ($p>0.05$). Again,

BRB9 and BRB10 analysis revealed RGD-mediated effects on migration but also an additional non-RGD-mediated effect of BSP. When the RGD migration response was examined in isolation with CBA4, we saw that it was largely αvβ3-mediated (Figure 8B). Also shown in Figure 8C is the ability of the αv and αvβ5 antibodies to block attachment of the MDA-231 cells to BRB 9, indicating that the RGD peptides are comparable to the recombinant BSP fragments. We confirmed the presence of these integrins on MDA-231 cells by flow cytometric analysis and found the αvβ3 integrin to be expressed at lower levels (Figure 9A). Therefore, the MDA-231 cells appear to utilize different integrins for different cellular responses to BSP-RGD sequences (αvβ5-mediated cell proliferation and attachment and αvβ3-mediated migration).

MDA-MB-231 selectants. To confirm and extend our study of the integrin-specific responses in MDA-231 cells, we used fluorescence-activated cell sorting (FACS) to select two subpopulations of MDA-231 cells, one enriched for αvβ3 integrin expression and the other for αvβ5 integrin expression (designated 231αvβ3 and 231αvβ5, respectively). The integrin profiles of these two sublines are shown in Figures 9B and 9C. The integrin enrichment of these subpopulations were stable in culture for up to 4 weeks as indicated by FACS analysis.

We repeated our previous experiments on the integrin-selected subpopulations and found that the 231αvβ3 cells had an increased migratory capacity when compared to their 231αvβ5 counterparts (Figure 10A), consistent with our previous blocking studies. The 231αvβ5 selectants showed no evidence of RGD-dependent migration, while the 231αvβ3 selectants showed increased RGD-dependent migration compared to parental cells (BRB9>BRB10), and this could be blocked most effectively with the αvβ3 antibody. 231αvβ5 cells showed a more pronounced proliferative response to CBA4 (compared to parental cells) on day 5 and this could be effectively attenuated by αv or αvβ5 but not by αvβ3 antibodies. In contrast, the 231αvβ3 subpopulation showed a very low initial proliferative response which was not blocked by αvβ3 (Figure 10B). This was consistent with the RGD-dependent proliferation of the 231αvβ5 selectants in response to BRB9 but not BRB10. Although not shown, day 3 proliferation responses were similar. Results from the adhesion experiments with the MDA-231 subpopulations were similar to those from proliferation

experiments, but more pronounced. While both 231αvβ3 and 231αvβ5 populations show increased cell attachment to the BSP-derived RGD peptides, attachment could be inhibited by the αvβ5 blocking antibody only in the 231αvβ5 cells (Figure 10C). Blocking antibodies to αvβ3 were unable to inhibit attachment of αvβ3 cells, again suggesting an alternative mechanism for BSP-mediated attachment and proliferation in the αvβ3 selectants. As previously shown, adhesion appeared to be RGD-dependent since attachment of both selectant populations to BRB10 was significantly diminished ($p<0.05$) as compared to BRB9. As with parental cells, GRGDSP was unable to induce significant migration, proliferation or attachment in these sublines, showing the BSP specificity of this RGD response. Overall, the results with these selected subpopulations confirm the differential αvβ3 and αvβ5 integrin usage for migration, proliferation and adhesion of MDA-MB-231 cells to BSP.

These studies have demonstrated that BSP could indeed invoke positive growth, attachment and migratory responses by breast carcinoma-derived cells, and that while the proliferative and adhesive responses appeared to be αvβ5-mediated, the αvβ3 integrin seemed to mediate the migratory response. In the case of migration, an additional non-RGD stimulus exists in BSP. These responses were initially established with purified rat BSP followed by more detailed studies using recombinant human BSP fragments expressed in *E. coli* and BSP-specific RGD-containing peptides. We found that the cyclic form of the BSP-RGD peptide conferred migratory, proliferative and adhesive properties comparable to the RGD component of full length rat BSP, indicating a significant role of the integrin binding RGD domain of BSP in these responses.

Two previous studies have associated the interaction of BSP and αv-containing integrins with bone metastasis activities. Adhesion of the MDA-231 cell to rat vertebral bone slices could be blocked by specific BSP-derived RGD peptides, suggesting that metastasizing breast cancer cells use BSP in order to attach to the bone matrix, and also that the BSP-specific sequences flanking the RGD domain are important in adhesive functions of BSP (30). Another study demonstrated that transfection of 293 cells with either αvβ3 or αvβ5 promoted cellular attachment to BSP (55). Our studies have revealed a novel proliferative response of MDA-231 HBC cells to BSP, as well as

cellular attachment and migratory responses. We have also demonstrated the interaction between the BSP-RGD domain and specific integrins in MDA-231 cell proliferation, migration, and adhesion, behaviors which may contribute to the role of BSP in metastasis. It is important to note, however, that a large non-RGD migration response to BSP was present, and this could also figure prominently in breast cancer cell metastasis.

Given the abundance of BSP in primary breast tumors as well as in bone, these breast cancer cell responses could be instrumental in both increased invasion of cells from the primary tumor, and in the successful establishment of bone metastasis. In addition, since BSP has been shown to be responsible for stimulating bone resorption (43), integrin-mediated attachment to bone matrix may also provide a direct mechanism for tumor cell osteolysis of bone. This is the first time that BSP has been reported to influence tumor cell proliferation and migration, broadening the scope of its involvement in breast cancer and bone metastasis, and to the best of our knowledge, this is also the first description of an interaction between the $\alpha v\beta 5$ integrin and BSP. These data imply that different cellular responses to the RGD domain of BSP may be regulated by separate populations of integrins, and that an interaction between breast cancer cells and the bone matrix may be important for bone metastasis.

C. Recommendations

The original statement of work submitted with this proposal listed five tasks, several of which we have already addressed, and the remainder of which we have begun or will shortly begin working on:

Task 1. Examine BSP and OPN mRNA and protein expression in breast cancer cell lines (*in vitro* as well as in different microenvironments *in vivo*).

a. We have examined BSP and OPN mRNA and protein expression in HBC cell lines *in vitro* and nude mouse xenografts (*in vivo)* (Northern analysis completed, with Western analysis in progress). However, we project difficulty in comparing BSP expression in bone metastatic HBC cell sublines

with those metastasizing to select soft organs since none of the HBC cell lines initially studied appear to express BSP *in vitro* or *in vivo*. Instead, we will compare BSP transfectants with their parental, non-BSP-expressing cells, both *in vivo* and *in vitro* (see below).

b. BSP and OPN immunoreactivity in HBC cell xenografts and the LCC15-MB bone-derived HBC cell line has been completed, as well as immunocytochemical analysis of HBC cell lines, paraffin embedded breast cancer specimens and associated bone material. A more detailed study of the LCC15-MB cell line has already been initiated. This study examines BSP and OPN expression by the cell line as well as the primary tumor, lymph node metastasis and bone metastasis from which it was derived in order to better characterize the molecular mechanisms of bone metastasis and to look for a possible correlation between bone metastasis the expression of the sialoproteins. In addition, we will characterize the invasive profile of the LCC15-MB cells and use them in our intracardiac injection model to directly assess bone metastasis *in vivo* in nude mice.

Task 2. Examine migratory and proliferative responses of HBC cells to BSP and OPN protein and peptides.

a. We have completed migration analysis of HBC cells toward BSP protein, recombinant BSP fragments and BSP-derived RGD peptides using the Boyden chamber model. Although not mentioned above, we have also begun characterizing the LCC15-MB and MDA-MB-435 HBC cell responses to BSP. Although OPN protein and a several recombinant fragments (but not peptides) have been made available to us, similar work appears to be in process at another laboratory, and for these reasons, we decided to concentrate primarily on cell responses to BSP and investigate, in detail, the domains required for these responses.

b. We have completed proliferation analyses of MDA-MB-231 cells toward BSP using 96 well plate assays and have begun to look at a few other HBC cell lines. Again, we have chosen to more closely study BSP responses and have delayed experiments with OPN.

c. Although not mentioned in the original statement of work, we have expanded these studies to include attachment profiles of the HBC cells to BSP and its derivatives. As described earlier in the results section, and in addition to migration and proliferation studies, we have analyzed MDA-MB-231 cell adhesion to BSP and localized a domain which may be responsible for this behavior.

Task 3. Investigate *in vivo* metastatic ability of HBC cells expressing BSP/OPN, especially those which are bone-derived.

a. As mentioned above, we did not find any HBC cell lines which express BSP (*in vitro* or *in vivo*), making it difficult to rationalize potential animal studies to study metastasis. However, there are two HBC cell lines which express abundant levels of OPN, and *in vivo* assays are currently under way to explore whether OPN promotes invasion and metastasis, either to the bone or other soft organs. When available, we will also test our BSP-expressing transfectants (see Task 4, below) for metastatic potential *in vivo*.

Task 4. Transfect cells with sense or antisense vectors to further examine the role of BSP and OPN in *in vivo* metastasis.

a. Based on our findings which showed lack of BSP expression in breast cancer cell lines, we have chosen to express sense vectors for BSP and OPN in the MDA-MB-231 HBC cell line which expresses neither of the sialoproteins. We have created expression vectors for both of these proteins and are currently in the process of selecting our transfectants. These clones will be tested in two *in vivo* assays (subcutaneous and intracardiac injection models) in order to explore the potential role(s) of BSP and OPN in HBC cell invasion and metastasis. We will also examine our transfectants in the *in vitro* assays to look for possible alterations in their migration, proliferation and adhesion profiles.

Task 5. Elucidate the domains of these proteins responsible for increased migratory and proliferative potential, as well as increased *in vivo* bone metastasis.

a. After determining positive migratory, proliferative and adhesive responses of an HBC cells to BSP, we used recombinant BSP fragments and the BSP-derived RGD peptides to elucidate the domain of the protein responsible for these behaviors (the RGD integrin-binding domain).

b. Because we determined the RGD domain to be responsible for migration, proliferation and adhesion responses, we used integrin blocking antibodies, rather than peptides, in order to block the BSP-induced responses of the HBC cell line. After identifying specific antibodies which blocked these responses, we also selected for subpopulations of the cell line which showed augmented

responses depending on augmented expression of a specific integrin. Since the responses could be blocked rather well with specific integrin blocking antibodies, we plan *in vivo* experiments using the integrin blockers. Both the intracardiac injection model and traditional nude mouse xenograft model will be utilized in order to examine whether the integrins play an important role in overall invasion and metastasis or, more specifically, in metastasis of HBC cells to the bone.

V. Conclusion

Although bone is the second most common organ in which breast cancer metastases develop, the molecular mechanisms responsible for preferential targeting of human breast cancer (HBC) cells to bone are not well understood. Previous research has shown that systemic as well as local factors appear to be involved. Recent reports in the literature which localized bone sialoprotein and osteopontin in primary breast lesions have suggested that they may be somehow involved in HBC invasion and the ability of certain breast cancer cells to metastasize to bone (5-7). Our studies have begun to explore the expression of BSP and OPN in HBC cells and several specific responses of cells to BSP which may aid in the process of tumor metastasis. In agreement with previous studies, we found expression of BSP and OPN in primary human breast lesions as well as their corresponding bone metastases. However, an in depth study of BSP mRNA and protein expression has failed to localize expression of this particular sialoprotein in any of the HBC cell lines examined. Two of the breast cancer cell lines we examined, MDA-MB-435 and LCC15-MB, did express considerable levels of OPN. Interestingly, they are two of the most invasive breast cancer cells, with the LCC15-MB cell being derived from a femoral bone metastasis of breast cancer. Consequently, we are exploring the possibility that OPN expression by primary breast cancer cells may help to promote their invasive and bone metastatic phenotypes.

We have also demonstrated that the bone matrix protein, BSP, can stimulate the migration, proliferation and adhesion of MDA-231 HBC cells through its RGD domain, and by employing specific integrin blockers, that these events occur through usage of $\alpha v\beta 3$ and $\alpha v\beta 5$. Given the abundance of BSP in primary breast tumors as well as in bone, these breast cancer cell responses could be instrumental in both increased invasion of cells from the primary tumor, and in the successful establishment of bone metastasis. In addition, since BSP has been shown to be responsible for stimulating bone resorption (43), integrin-mediated attachment to bone matrix may also provide a direct mechanism for tumor cell osteolysis of bone. In summary, our findings thus far support the hypothesis that BSP and OPN play a role in the progression of breast cancer. It is still unknown if the invasion process simply requires the ability of a tumor cell to respond to these bone

matrix proteins (paracrine mechanism), and/or if inherent expression (autocrine mechanism) results in a more selective metastasis to bone. Also, the bone environment may induce cancer cells to express certain bone matrix proteins to further facilitate metastatic colonization and stimulation of osteolysis. Our ongoing studies will help to further clarify the mechanism(s) through which BSP and OPN act to promote tumor cell invasion, and will be potentially important in the future of breast cancer diagnosis and prevention/treatment of both overall tumor metastasis and bone metastatic disease.

VI. References

1.Yoneda T, Sasaki A, Mundy GR. 1994. Osteolytic bone metastasis in breast cancer. Breast Canc Res Treat, 32: 73-84.

2. Mundy GR. 1990. Pathophysiology of cancer-associated hypercalcemia. Semi Oncol, 17: 10-15.

3. Orr FW, Kostenuik P, Sanchez-Sweatman OH, Singh G. 1993. Mechanisms involved in the metastasis of breast cancer to bone. Breast Cancer Res Treat, 25: 151-163.

4. Mohan S, Baylink DJ. 1991. Bone growth factors. Clin Orthop Rel Res, 236: 30-48.

5. Bellahcene A, Merville MP, Castronovo V. 1994a. Expression of bone sialoprotein, a bone matrix protein, in human breast cancer. Cancer Res, 54: 2823-2826.

6. Bellahcene, A., and Castronovo, V. Increased expression of osteonectin and osteopontin, two bone matrix proteins, in human breast cancer. 1994b. Am J Path, 146: 95-100.

7. Bellahcene A, Kroll M, Liebens F, Castronovo V. 1996. Bone sialoprotein expression in primary human breast cancer is associated with bone metastasis development. J Bone Mineral Res, 11: 666-670.

8. Brown LF, Papadopoulos-Sergiou A, Berse, B, Manseau EJ, Tognazzi K, Perruzzi CA, Dvorak HF, Senger DR. 1994. Osteopontin expression and distribution in human carcinomas. Am J Pathol, 145: 610-623.

9. Hirota S, Ito, A, Nagoshi J, Takeda M, Kurata A, Takatsuka Y, Kohri K, Nomura S, Kitamura Y. 1995. Expression of bone matrix protein messenger ribonucleic acids in human breast cancers. Lab Invest, 72: 64-69.

10. Fisher LW, Whitson SW, Avioli LV, Termine JD. 1983. Matrix sialoprotein of developing bone. J Biol Chem, 258: 12723-12727.

11. Bianco P, Fisher LW, Young MF, Termine JD, Robey PG. 1991. Expression of bone sialoprotein (BSP) in developing human tissues. Calcif Tissue Int, 49: 421-426.

12. Zhou H-Y, Takita H, Fujisawa R, Mizuno M, Kuboki Y. 1995. Stimulation of bone sialoprotein of calcification in osteoblast-like MC3T3-E1 cells. Calcif Tissue Int, 56: 403-407.

13. Hunter GK and Goldberg HA. 1993. Nucleation of hydroxyapatite by bone sialoprotein. Proc Natl Acad Sci USA, 90: 8562-8565.

14. Oldberg A, Franzen A, Heinegard D. 1986. Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell-binding sequence. Proc Natl Acad Sci USA, 83: 8819.

15. Oldberg A, Franzen A, Heinegard D. 1988a. The primary structure of a cell binding bone sialoprotein. J Biol Chem, 263: 19430-19432.

16. Fisher LW, McBride OW, Termine JD, Young MF. 1990. Human bone sialoprotein: deduced protein sequence and chromosomal localization. J Biol Chem, 265: 2347-2351.

17. Chenu C, Ibaraki K, Geron Robey P, Delmas PD, Young MF. 1994. Cloning and sequence analysis of bovine bone sialoprotein cDNA: Conservation of acidic domains, tyrosine sulfation consensus repeats and RGD cell attachment domain. J Bone Mineral Res, 9: 417-421.

18. Young MF, Ibaraki K, Kerr JM, Lyu MS, Kozak CA. 1994. Murine bone sialoprotein (BSP): cDNA cloning, mRNA expression and genetic mapping. Mamm Genome, 5: 108-111.

19. Shapiro HS, Chen J, Wrana JL, Zhang Q, Blum M, Sodek J. 1993. Characterization of porcine bone sialoprotein: primary structure and cellular expression. Matrix, 13: 431-440.

20. Oldberg A, Franzen A, Heinegard D, Peirschbacher M, Ruoslahti E. 1988b. Identification of a bone sialoprotein receptor in osteosarcoma cells. J Biol Chem, 263: 19433-19436.

21. Helfrich MH, Nesbitt SA, Dorey EL, Horton MA. 1992. Rat osteoclasts adhere to a wide range of RGD (Asp-Gly-Asp) peptide-containing proteins, including the bone sialoproteins and fibronectin, via a beta 3 integrin. J Bone Mineral Res, 7: 335-343.

22. Ross FP, Chappel J, Alvarez JI, Sander D, Butler WT, Farach-Carson MC, Mintz KA. 1993. Interactions between the bone matrix proteins osteopontin and bone sialoprotein and the osteoclast integrin alpha v beta 3 potentiate bone resorption. J Biol Chem, 268: 9901-9907.

23. Fujisawa R, Kuboki Y. 1992. Affinity of bone sialoprotein and several other bone and dentin acidic proteins to collagen fibrils. Calcif Tiss Int, 51: 438-442.

24. Chellaiah M, Fitzgerald C, Filrado EJ, Cheresh DA, Hruska KA. 1996. Osteopontin activation of c-src in human melanoma cells requires the cytoplasmic domain of the integrin αv-subunit. Endocrinology, 137: 2432-2440.

25. Bautista DS, Xuan J-W, Hota C, Chambers AF, Harris JF. 1994. Inhibition of Arg-Gly-Asp (RGD)-mediated cell adhesion to osteopontin by a monoclonal antibody against osteopontin. J Biol Chem, 37: 23280-23285.

26. Xuan J-W, Hota C, Shigeyama Y, D'Errico JA, Somerman MJ, Chambers AF. 1995. Site-

directed mutagenesis of the arginine-glycine-aspartic acid sequence in osteopontin destroys cell adhesion and migration functions. J Cell Biochem, 57: 680-690.

27. Van Dijk S, D'Errico JA, Somerman MJ, Farach-Carson MC, Butler WT. 1993. Evidence that a non-RGD domain in rat osteopontin is involved in cell attachment. J Bone Mineral Res, 8: 1499-1506.

28. Stubbs JT III, Eanes ED, Fisher LW. 1995. The use of native and recombinant fragments to delineate non-RGD cell attachment domains and mineral-binding domains of bone sialoprotein. J Bone Mineral Res, 10(S1): S432.

29. Mintz KP, Grzesik WJ, Midura RJ, Robey PG, Termine JD, Fisher LW. 1993. Purification and fragmentation of nondenatured bone sialoprotein: evidence for cryptic, RGD-resistant cell attachment domain. J Bone Mineral Res, 8: 985-995.

30. Van der Pluijm G, Vloedgraven HJM, Ivanov B, Robey F, Grezesik WJ, Robey PG, Papapolous SE, Lowik CWGM. 1996. Bone sialoprotein peptides are potent inhibitors of breast cancer adhesion to bone. Canc Res, 56: 1948-1955.

31. Sheppard D. 1996. Epithelial integrins. Bioessays, 18: 655-660.

32. Clark EA, Brugge JS. 1995. Integrins and signal transduction pathways: the road taken. Science, 268: 233-238.

33. Zutter MM, Krigman HR, Santoro SA. 1993. Altered integrin expression in adenocarcinoma of the breast. Anaylysis by *in situ* hybridization. Am J Pathol, 142: 1439-1448.

34. Zutter MM, Santoro SA, Staatz WD, Tsung YL. 1995. Re-expression of the $\alpha 2\beta 1$ integrin abrogates the malignant phenotype of breast carcinoma cells. Proc Natl Acad Sci USA, 92: 7411-7415.

35. Yun Z, Menter DG, Nicolson GL. 1996. Involvement of integrin $\alpha v\beta 3$ in cell adhsion, motility, and liver metastasis of murine RAW117 large cell lymphoma. Cancer Res, 56: 3103-3111.

36. Montgomery AM, Reisfeld RA, Cheresh DA. 1994. Integrin $\alpha v\beta 3$ rescues melanoma cells from apoptosis in three-dimensional dermal collagen. Proc Natl Acad Sci USA, 91: 8856-8860.

37. Brooks PC, Clark RA, Cheresh DA. 1994. Requirement of vascular integrin alpha v beta 3 for angiogenesis. Science, 264: 560-571.

38. Brooks PC, Montgomery AM, Rosenfeld M, Reisfeld RA, Hu T, Klier G, Cheresh DA. 1994. Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic

blood vessels. Cell, 79: 1157-1164.

39. Davies J, Warwick J, Totty N, Philip R, Helrich M, Horton M. 1989. The osteoclast functional antigen, implicated in the regulation of bone resorption, is biochemically related to the vitronectin receptor. J Cell Biol, 109: 1817-1826.

40. Horton MA and Davies J. 1989. Perspectives: adhesion receptors in bone. J Bone Mineral Res, 4: 803-808.

41. Kostenuik PJ, Sanchez-Sweatman O, Orr FW, Singh G. 1996. Bone cell matrix promotes the adhesion of human prostatic caricinoma cells via the $\alpha 2\beta 1$ integrin. Clin Exp Metas, 14: 19-26.

42. Liapis H, Flath A, Kitazawa S. 1996. Integrin $\alpha v\beta 3$ expression by bone-residing breast cancer metastasis. Diag Molec Path, 5: 127-135.

43. Raynal, C., Delmas, P.D., and Chenu, C. 1996. Bone sialoprotein stimulates *in vitro* bone resorption. Endocrinol *137*: 2347-2354.

44. Thompson, EW, Sung, V, Lavigne, M, Baumann, K, Azumi, N, Aaron, AD and Clarke, R. LCC15-MB: A Human Breast Cancer Cell Line from a Femoral Bone Metastasis. 1997. Abstract, AACR meeting, San Diego, CA.

45. Mintz, K.P., Midura, R.J., Robey, P.G., and Fisher, L.W. 1994. Purification of bone sialoprotein from the medium of the rat osteoblast-like cell line UMR 106-01BSP. J Tissue Cult Methods *16*: 2347-2351.

46. Midura R, McQuillan DJ, Benham KJ, Fisher LW, Hascall VC. 1990. A rat osteogenic cell line (UMR106-01) synthesizes a highly sulfated form of bone sialoprotein. J Biol Chem, 265: 5285.

47. Stubbs JT. 1996. Generation and use of recombinant human bone sialoprotein and osteopontin for hydroxyapatite studies. Connective Tiss Res, in press.

48. Chomczynski P, Sacchi N. 1987. Single step method of RNA isolation by acid guanidinium thiocynate-phenol-chloroform extraction. Anal Biochem, 162: 156.

49. Young MF, Kerr JM, Termine JD, Wewer UM, Wang MF, McBride OW. 1990. cDNA cloning, mRNA distribution and heterogeneity, chromosomal location, and RFLP analysis of human osteopontin (OPN). Genomics, 7: 491.

50. Fisher LW, Termine JD, Young MF. 1989. Deduced protein sequence of bone small proteoglycan I (Biglycan) shows homology with proteoglycan II (Decorin) and several nonconnective tissue proteins in a variety of species. J Biol Chem, 264: 4571.

51. Thompson, E.W., Paik, S., Brunner, N., Sommers, C.L., Zugmaier, G., Clarke, R., Shima, T.B., Torri, J., Donahue, S., Lippman, M.E., Martin, G.E., and Dickson, R.B. 1992. Association of increased basement membrane-invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. J Cell Physiol *150*: 534-544.

52. Thompson, E.W., Brunner, N., Torri, J., Boulay, V., Wright, A., Steeg, P.S., Lippman, M.E., and Clarke, R. 1993. The invasive and metastatic properties of hormone-independent but hormone-responsive variants of MCF-7 human breast cancer cells. Clin Exp Metas *11*: 15-26.

53. Oates, A.J., Barraclough, R., and Rudland, P.S. 1996. The identification of osteopontin as a metastasis-related gene product in a rodent mammary tumor model. *Oncogene*, 13: 97-104.

54. Bernstein, L.R., and Liotta, L.A. Molecular mediators of interactions with extracellular matrix components in metastasis and angiogenesis. 1994. Curr Opin Oncol, *6*: 106-113.

55. Duong, L.T., Nutt, E.M., and Rodan, G.A. Distinct interactions of $\alpha v\beta 3$ and $\alpha v\beta 5$ vitronectin receptors with extracellular matrix proteins. 1996. Abstract ASBMR 18th annual meeting: M433.

VII. Appendices

Figure 1

Schematic depiction of full length rat bone sialoprotein (BSP) and the different human BSP-derived constructs used in this study. BRB9 and BRB10 are recombinant fragments of human BSP with and without (KAE substituted for RGD) the RGD domain, respectively, and CBA4 and BA3 are the cyclic and linear forms of the human BSP-derived RGD peptide.

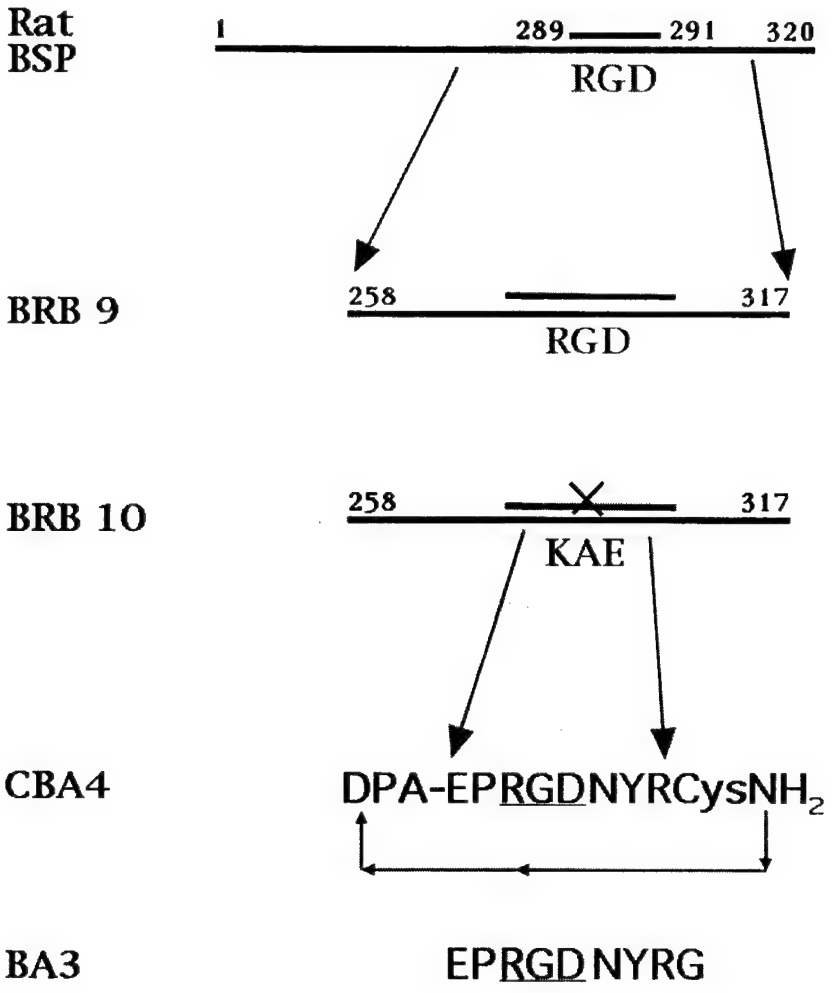

Rat BSP
1
289
291
320
RGD
BRB 9
258
317
RGD
BRB 10
258
317
KAE
CBA4
DPA-EPRGDNYRCysNH2
BA3
EPRGDNYRG

Figure 2

Expression of BSP in randomly selected primary breast malignancies **(A,B)** and a primary malignancy **(C)** and its associated bone metastasis **(D)** as assayed by immunocytochemistry using a polyclonal antibody against BSP (LF83). These four paraffin-embedded sections have also been counterstained using hematoxylin. Magnification = 10X.

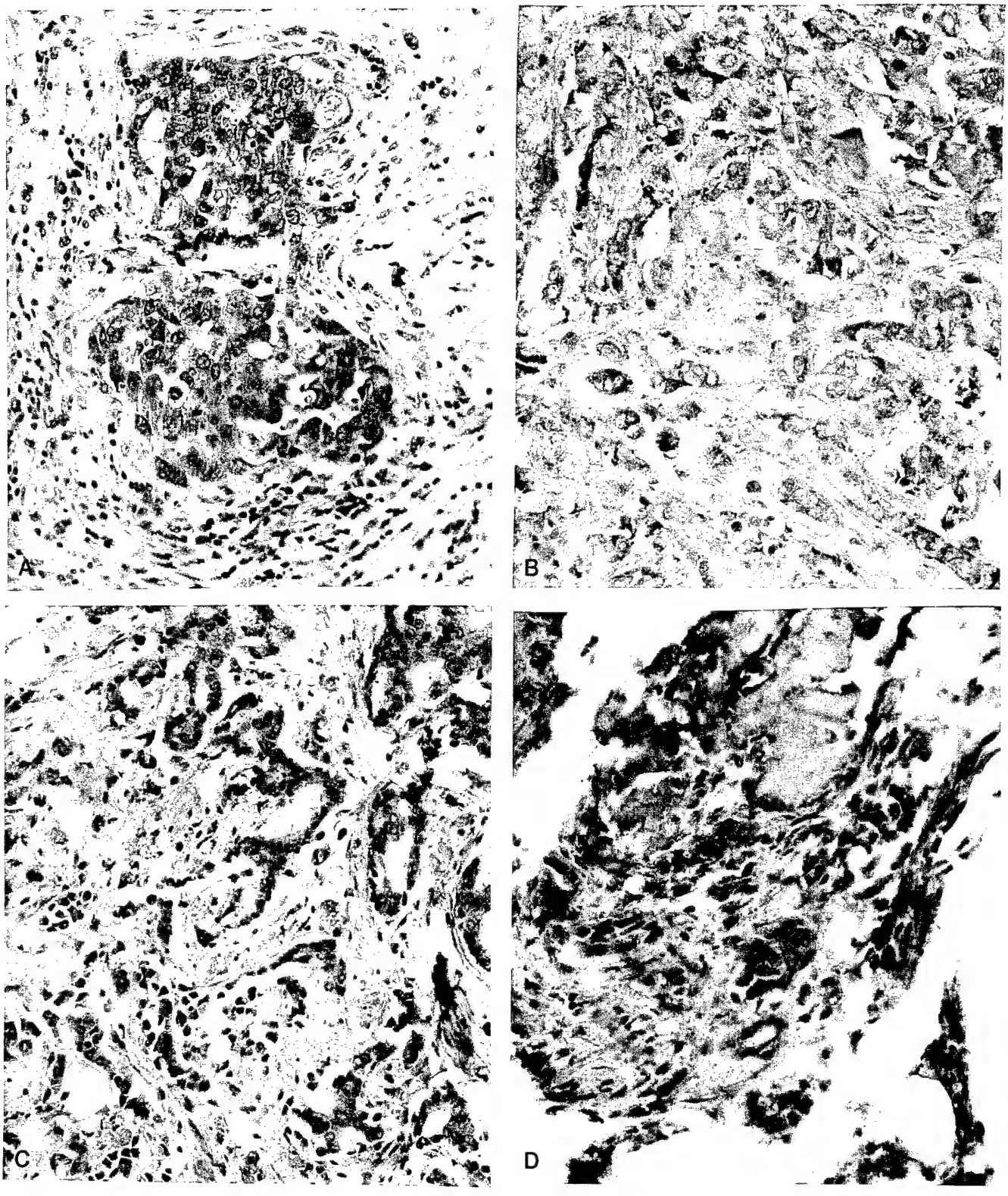
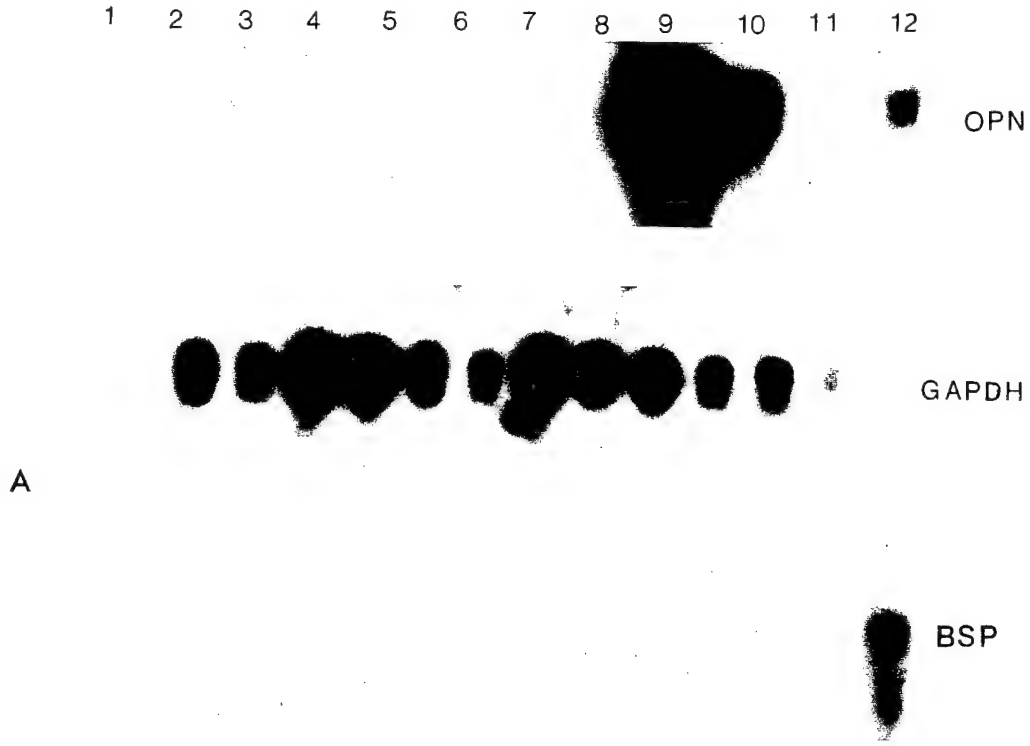

A
B
C
D

Figure 3

Expression of BSP and OPN mRNA in (**A**) various HBC cell lines (Lane 1-MCF-7, 2-T47D, 3-MCF-7_{ADR}, 4- SKBr3, 5-MDA-MB-453, 6- BT549, 7- MDA-MB-436, 8-MDA-MB-231, 9-MDA-MB-435, 10- LCC15-MB, 11-Hs578T, 12- UMR-106-01BSP) and nude mouse xenografts (**B**) (Lanes 1,2: UMR-106-01BSP, 4,5-LCC15-MB, 7,8-MDA-MB-435, 10,11- UMR-106-01) as assayed by Northern blotting. RNA loading was normalized using a GAPDH control. The UMR-106-01BSP rat osteosarcoma cell line which expresses endogenous BSP and OPN was used as a positive control in both Northern blots.


1 2 3 4 5 6 7 8 9 10 11 12
OPN
GAPDH
A

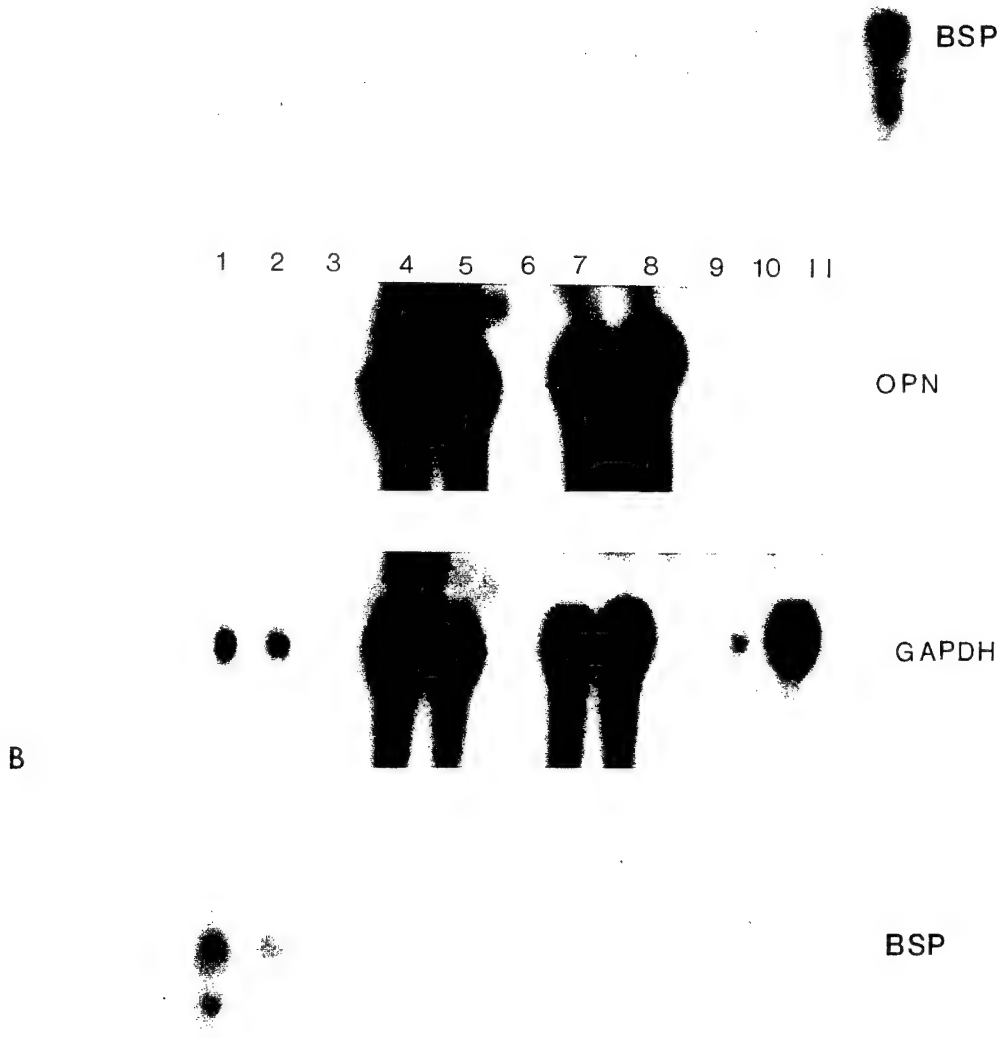
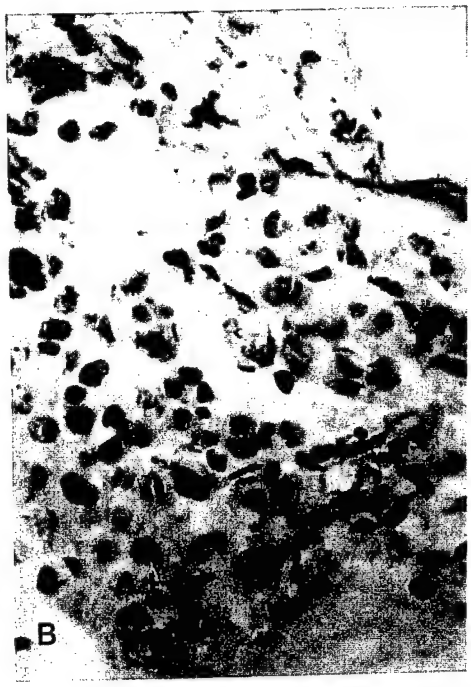
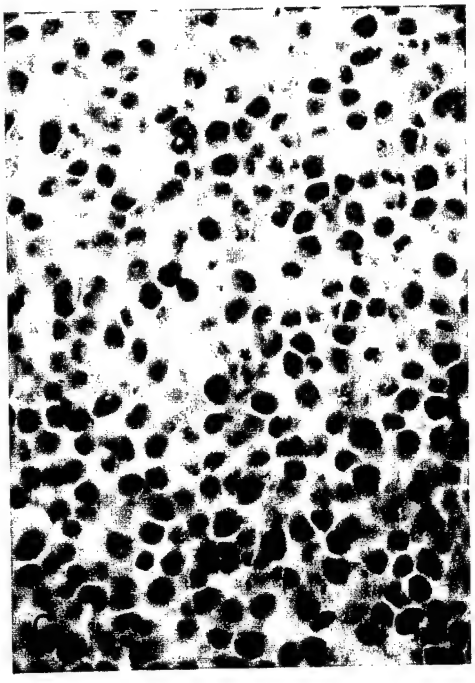
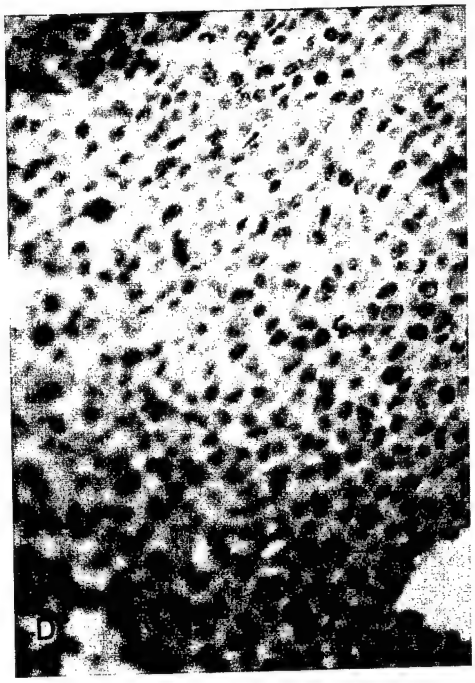

BSP
1 2 3 4 5 6 7 8 9 10 11
OPN
GAPDH
B
BSP

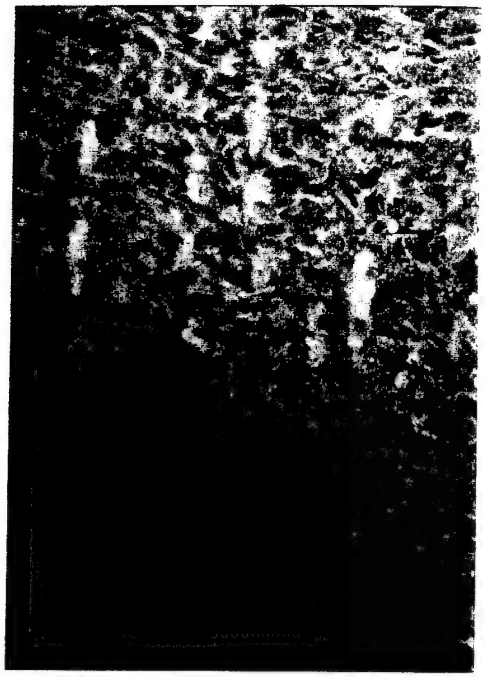
Figure 4

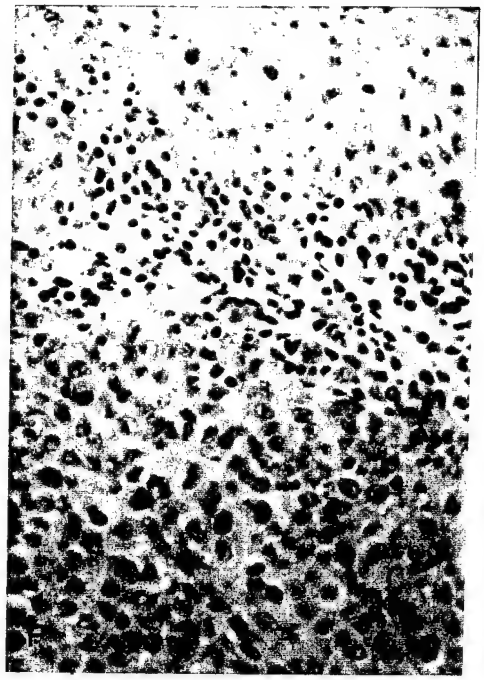
Expression of BSP in UMR-106-01BSP, MDA-MB-231, MDA-MB-435 and LCC15-MB HBC cell line plugs **(A-D)** and nude mouse xenografts **(E-H)** as assayed by immunocytochemistry using a polyclonal antibody to BSP (LF83). Magnification = 50X.


B




D





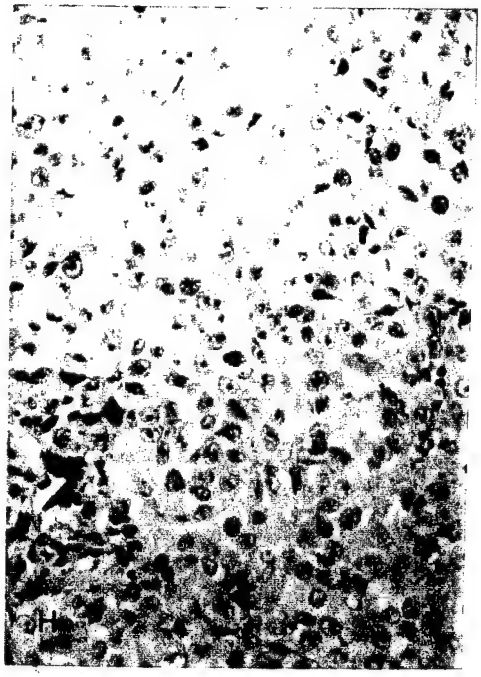
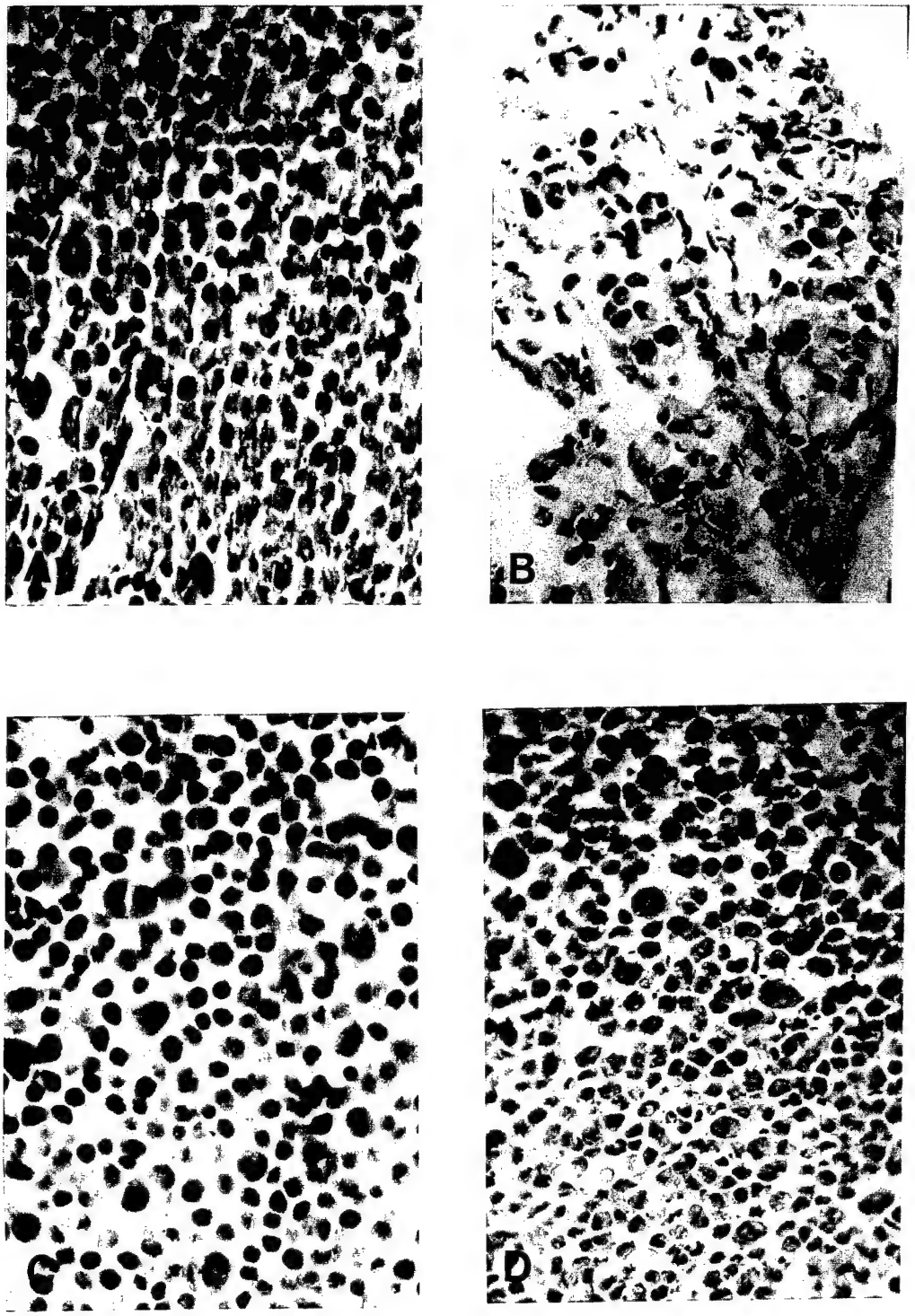
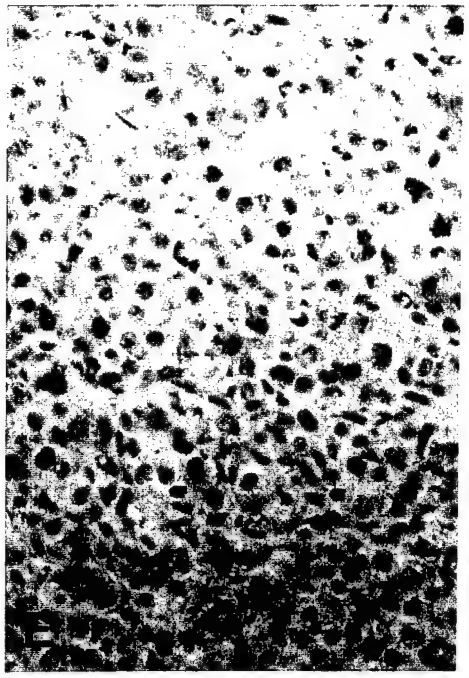


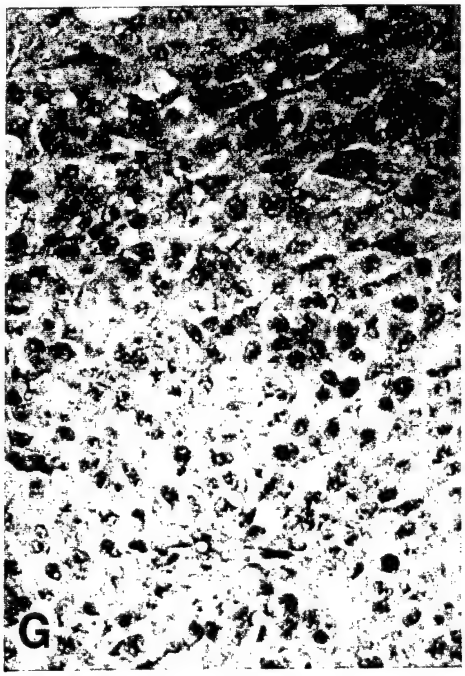
Figure 5

Expression of OPN in UMR-106-01BSP, MDA-MB-231, MDA-MB-435 and LCC15-MB HBC cell line plugs **(A-D)** and nude mouse xenografts **(E-H)** as assayed by immunocytochemistry using a polyclonal antibody to OPN (LF19). Magnification = 50X.


B
C
D


E


F


G

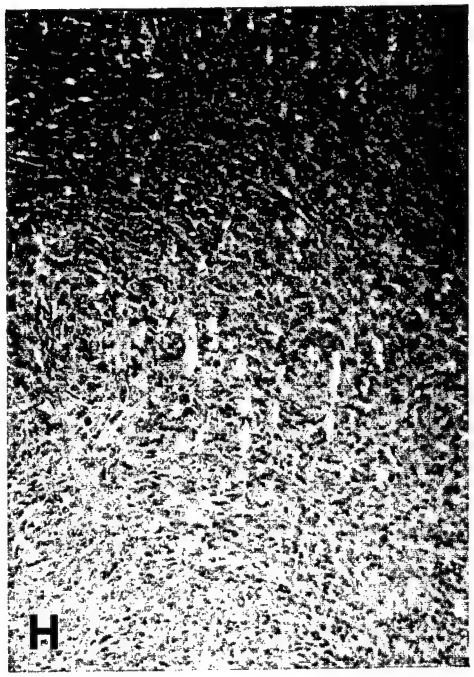
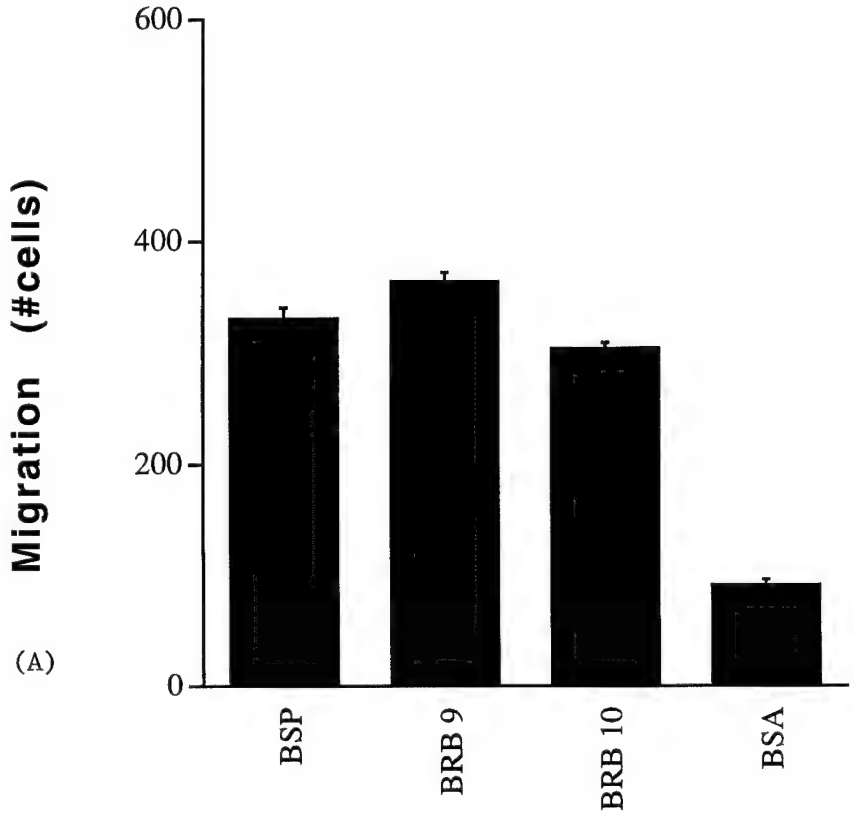
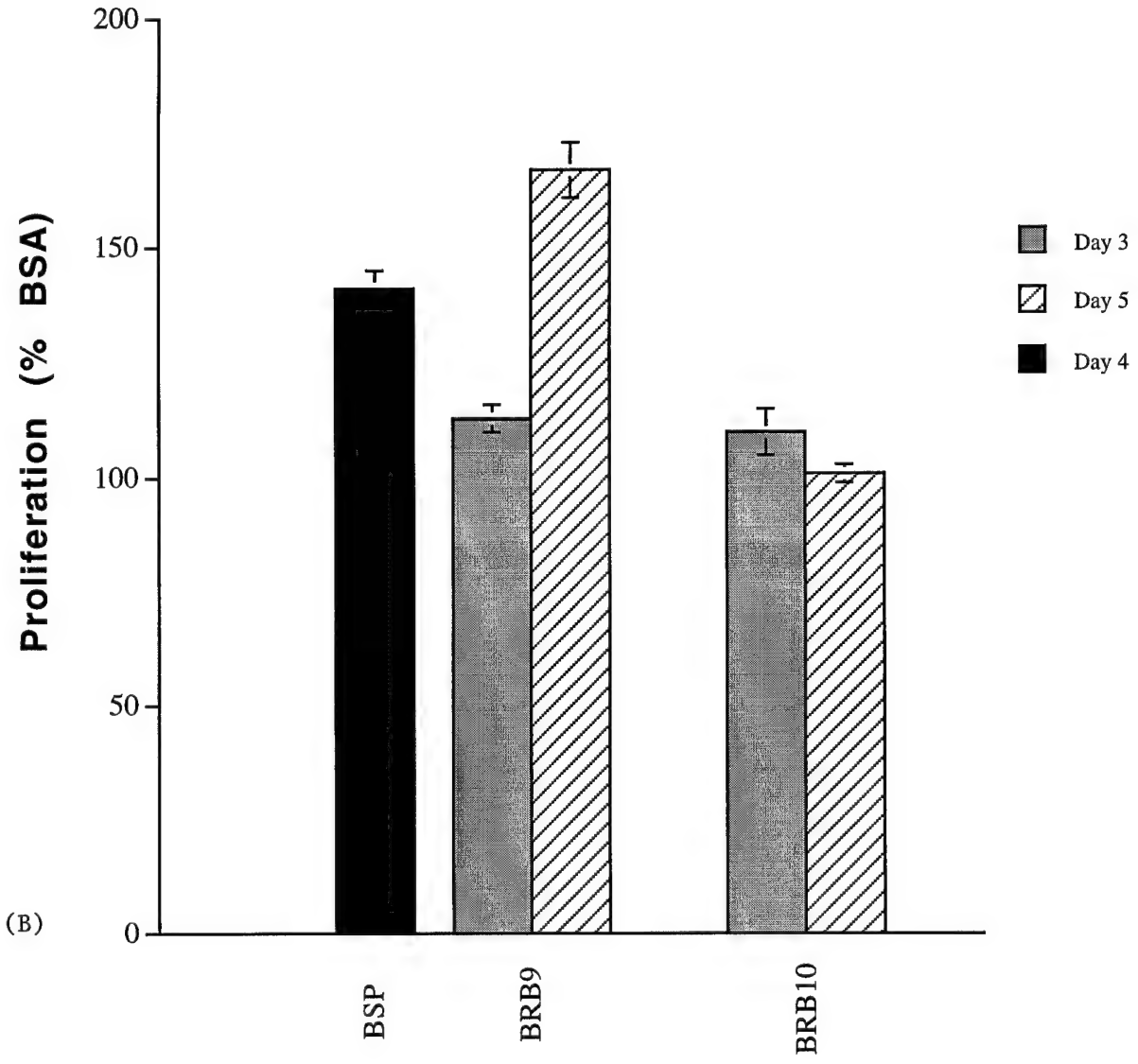

H

Figure 6

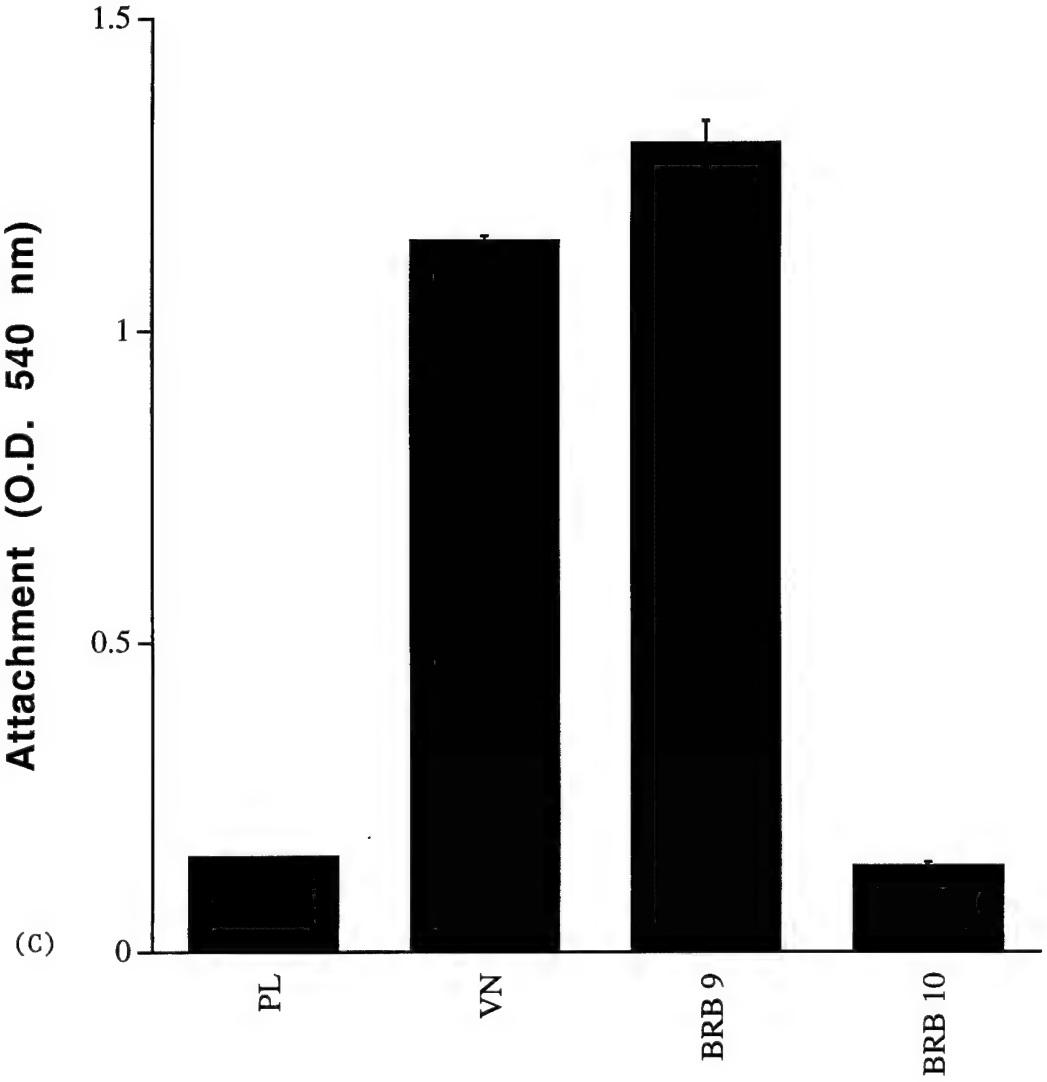
MDA-231 cell migration **(A),** proliferation **(B),** and attachment **(C)** to rat BSP and recombinant human BSP fragments BRB9 and BRB10. BSA (bovine serum albumin), PL (plastic) and VN (vitronectin). Bars represent mean and standard deviation from triplicate filters/wells in a representative experiment.

(A)




Proliferation (% BSA)
200
150
100
50
0
BSP
BRB9
BRB10
Day 3
Day 5
Day 4

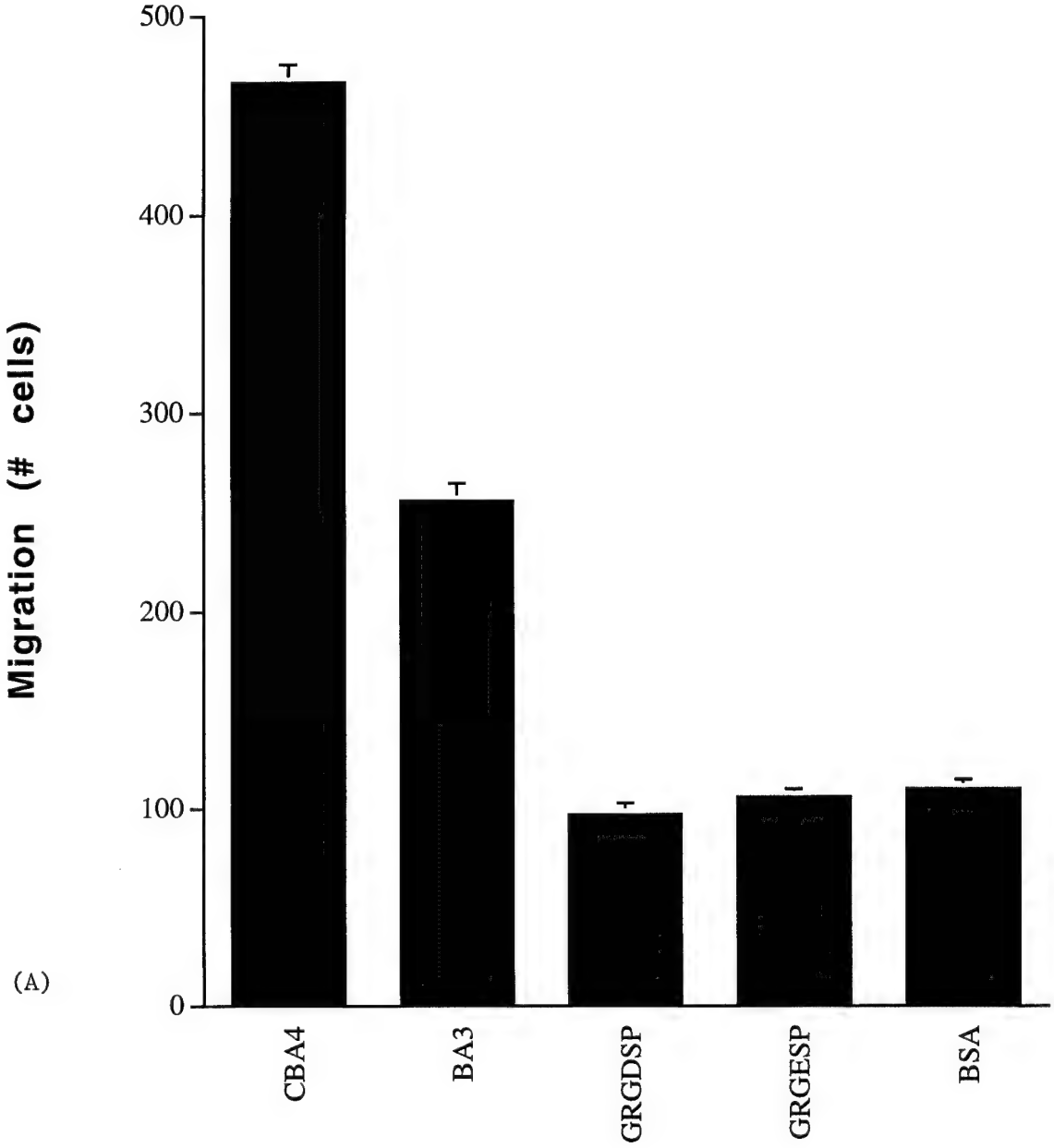
(B)



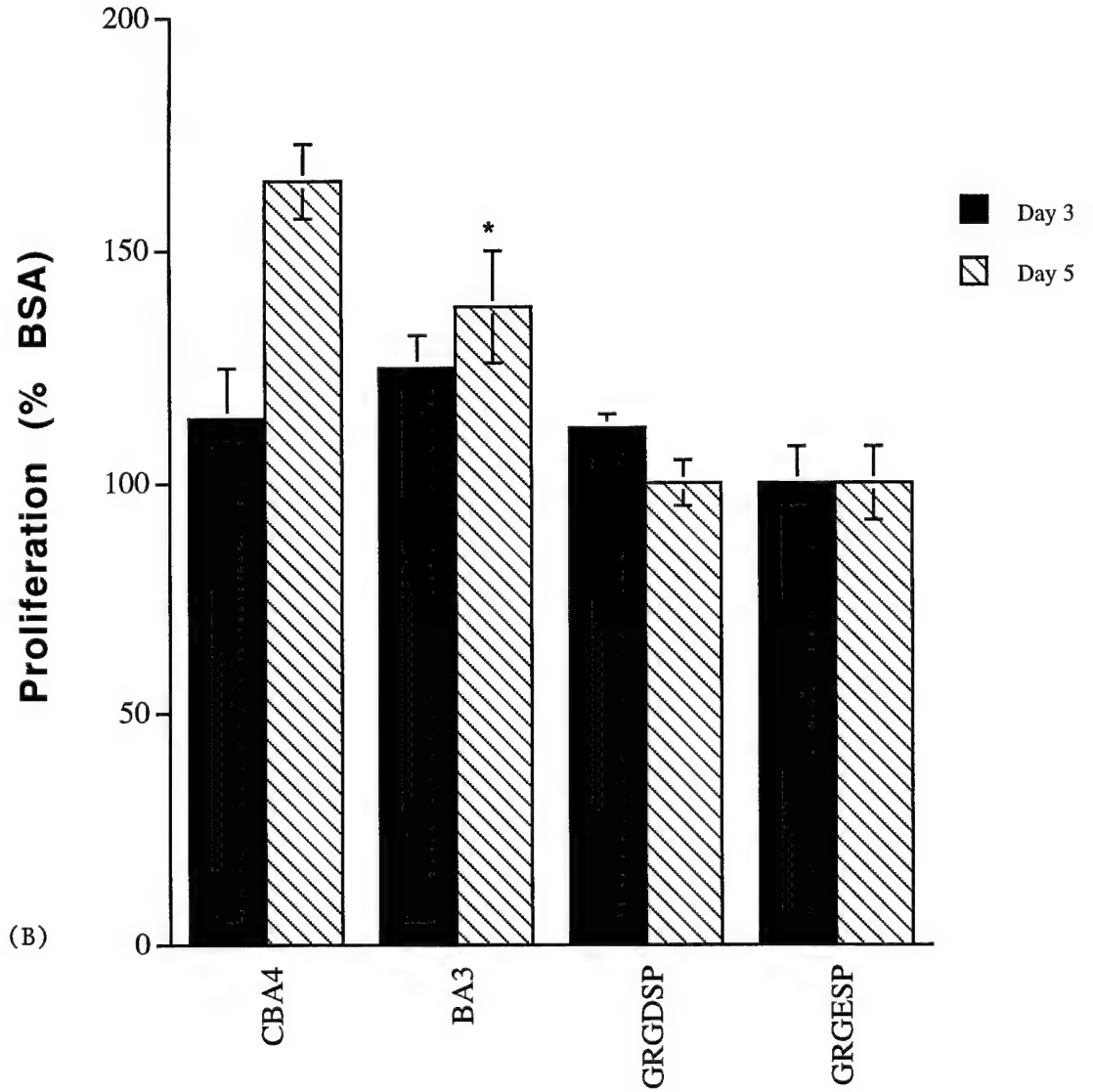
(C)

Figure 7

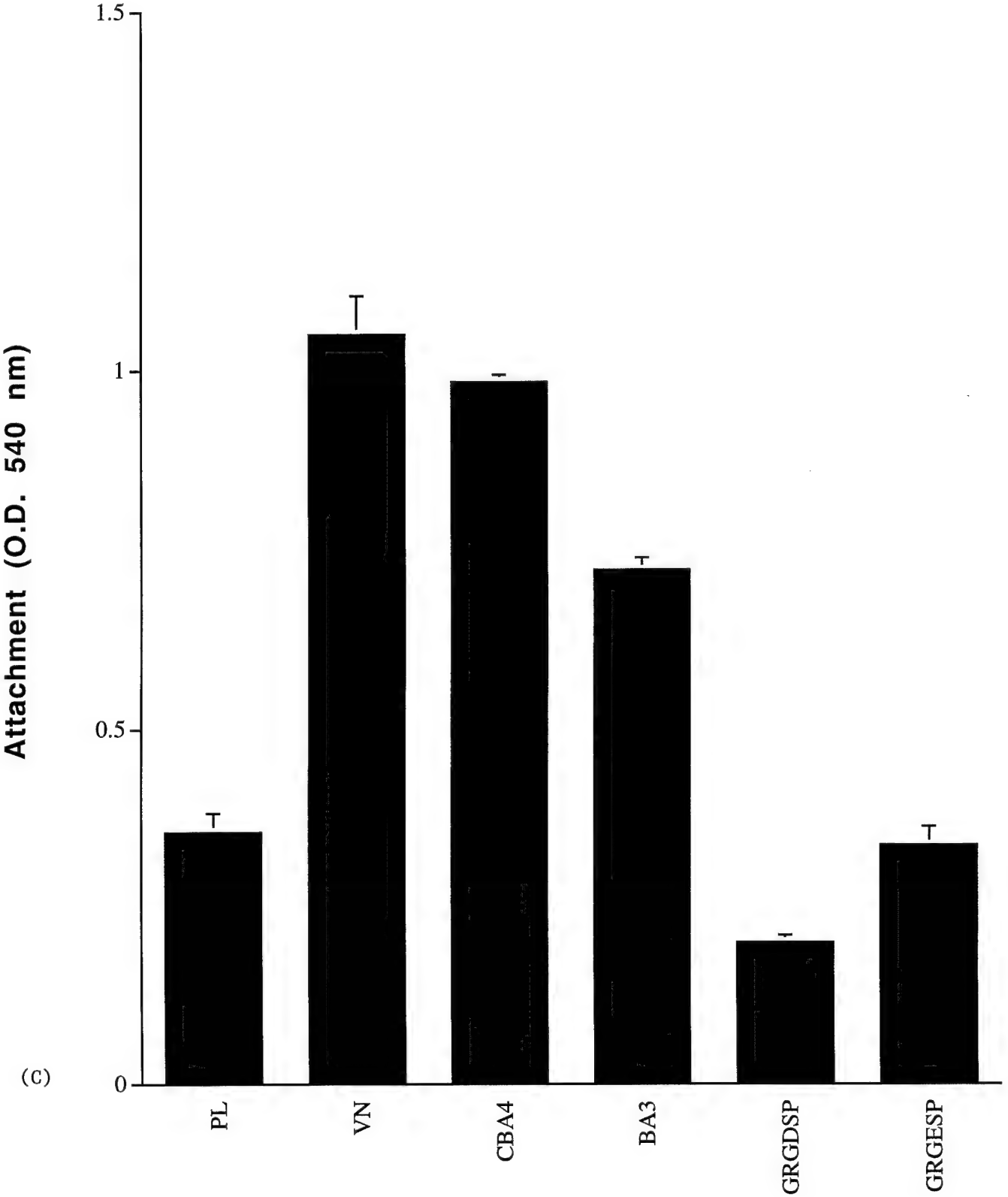
MDA-231 cell migration **(A),** proliferation **(B),** and attachment **(C)** to BSP-derived RGD peptides CBA4 and BA3, the fibronectin-derived RGD peptide (GRGDSP) and its corresponding mutant form (GRGESP). BSA (bovine serum albumin), PL (plastic) and VN (vitronectin). Bars represent mean and standard deviation from triplicate filters/wells in a representative experiment, and proliferation to BA3 is significantly lower than that obtained by CBA4 (day 5). *$P<0.05$ in two-sided t-test.


Migration (# cells)
500
400
300
200
100
0
CBA4
BA3
GRGDSP
GRGESP
BSA

(A)


Proliferation (% BSA)
200
150
100
50
0
*
Day 3
Day 5
CBA4
BA3
GRGDSP
GRGESP

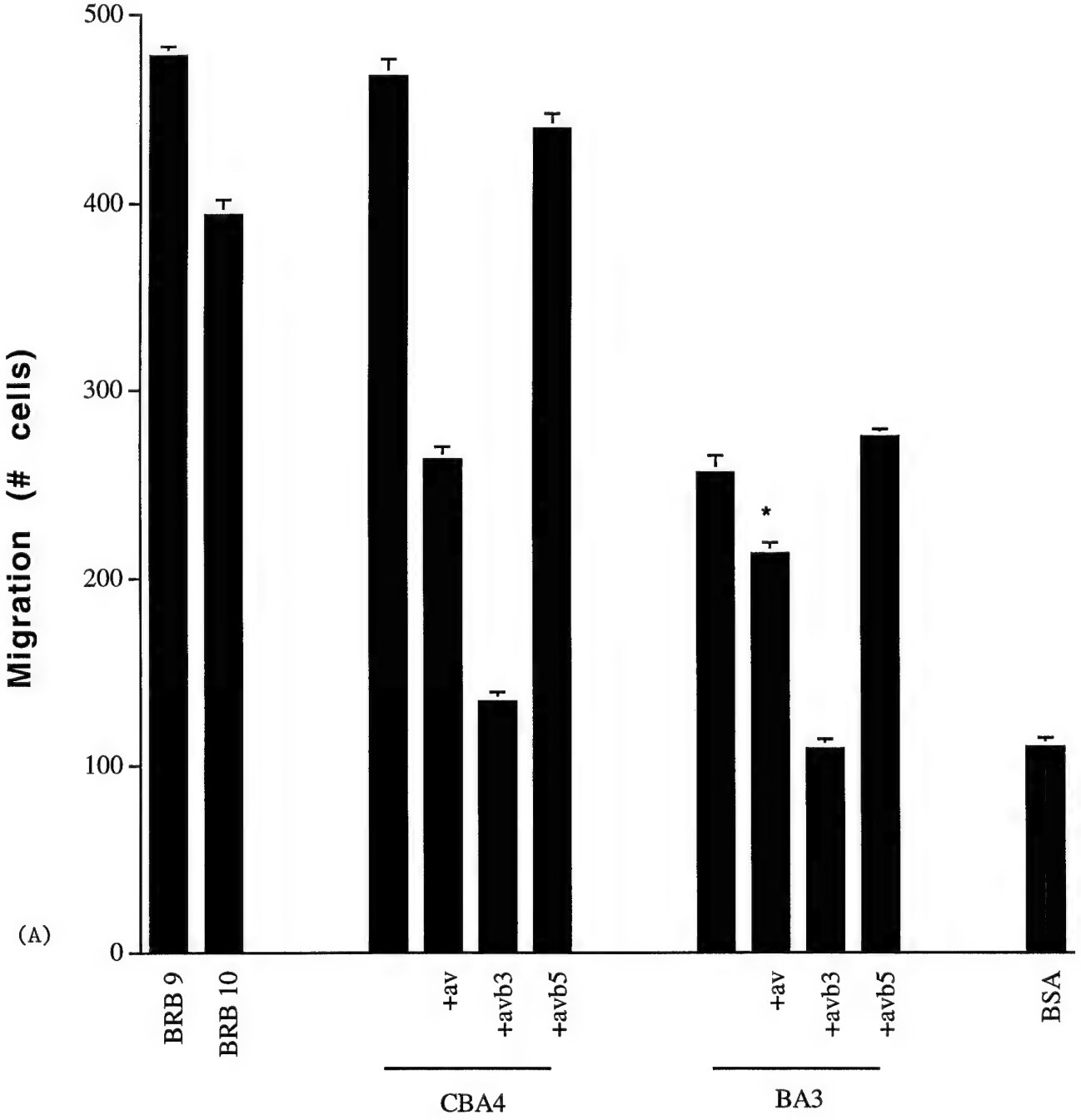
(B)



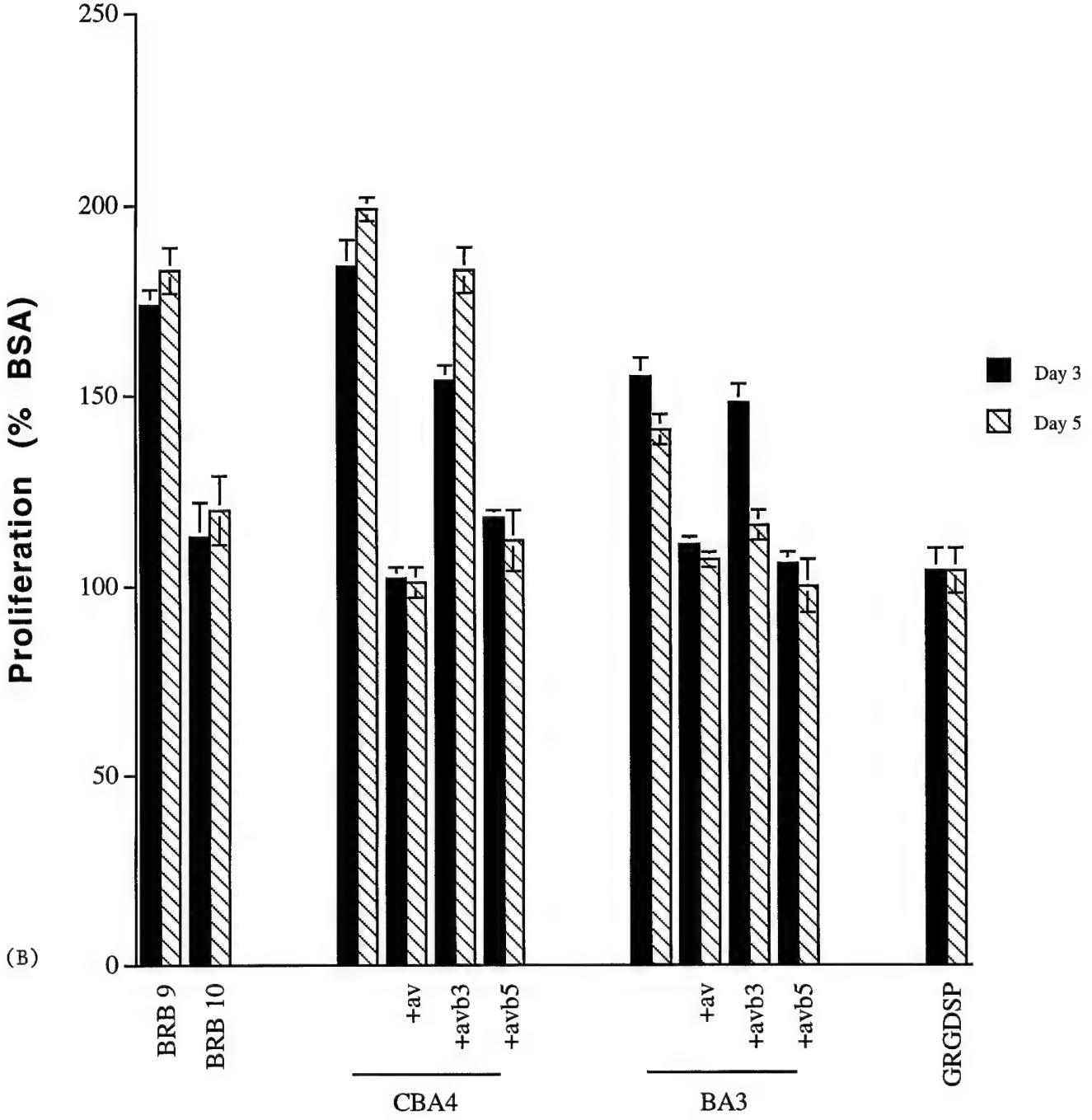
(C)

Figure 8

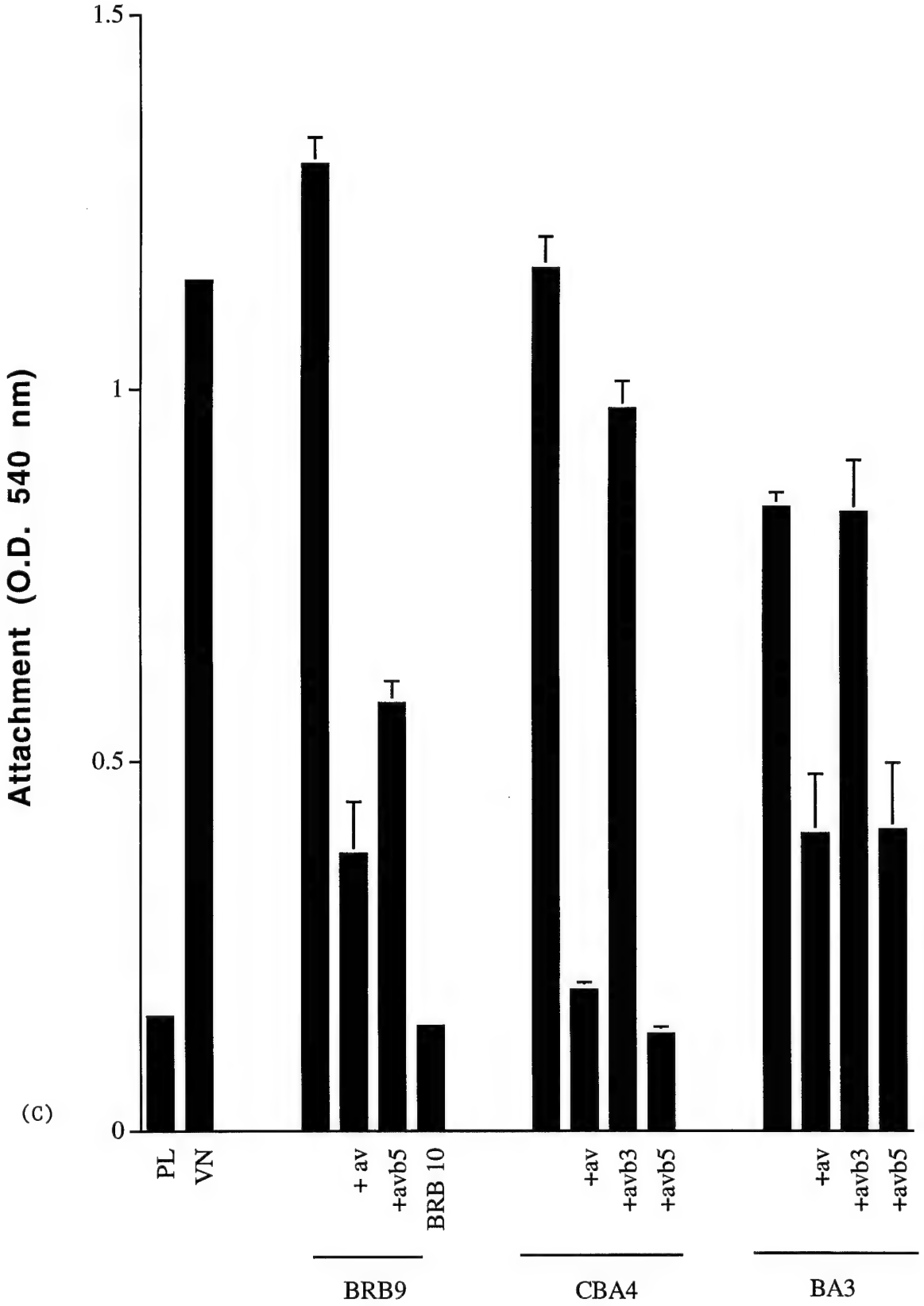
The effects of αv, αvβ3 and αvβ5 integrin blocking antibodies on MDA-231 cell migration **(A),** proliferation **(B),** and attachment **(C)** to BSP-derived RGD peptides CBA4 and BA3. Recombinant BSP fragments BRB9 and BRB10 are also shown as reference points. BSA (bovine serum albumin), GRGDSP (mutated fibronectin-derived RGD peptide), PL (plastic),and VN (vitronectin). Bars represent mean and standard deviation from triplicate filters/wells of a representative experiment, and αv-blocked migration to BA3 is significantly lower than migration toward BA3 alone. *$P<0.05$ in two-sided t-test.


Migration (# cells)
500
400
300
200
100
0
*
BRB 9
BRB 10
+av
+avb3
+avb5
+av
+avb3
+avb5
BSA
CBA4
BA3

(A)


Proliferation (% BSA)
250
200
150
100
50
0
Day 3
Day 5
BRB 9
BRB 10
+av
+avb3
+avb5
+av
+avb3
+avb5
GRGDSP
CBA4
BA3

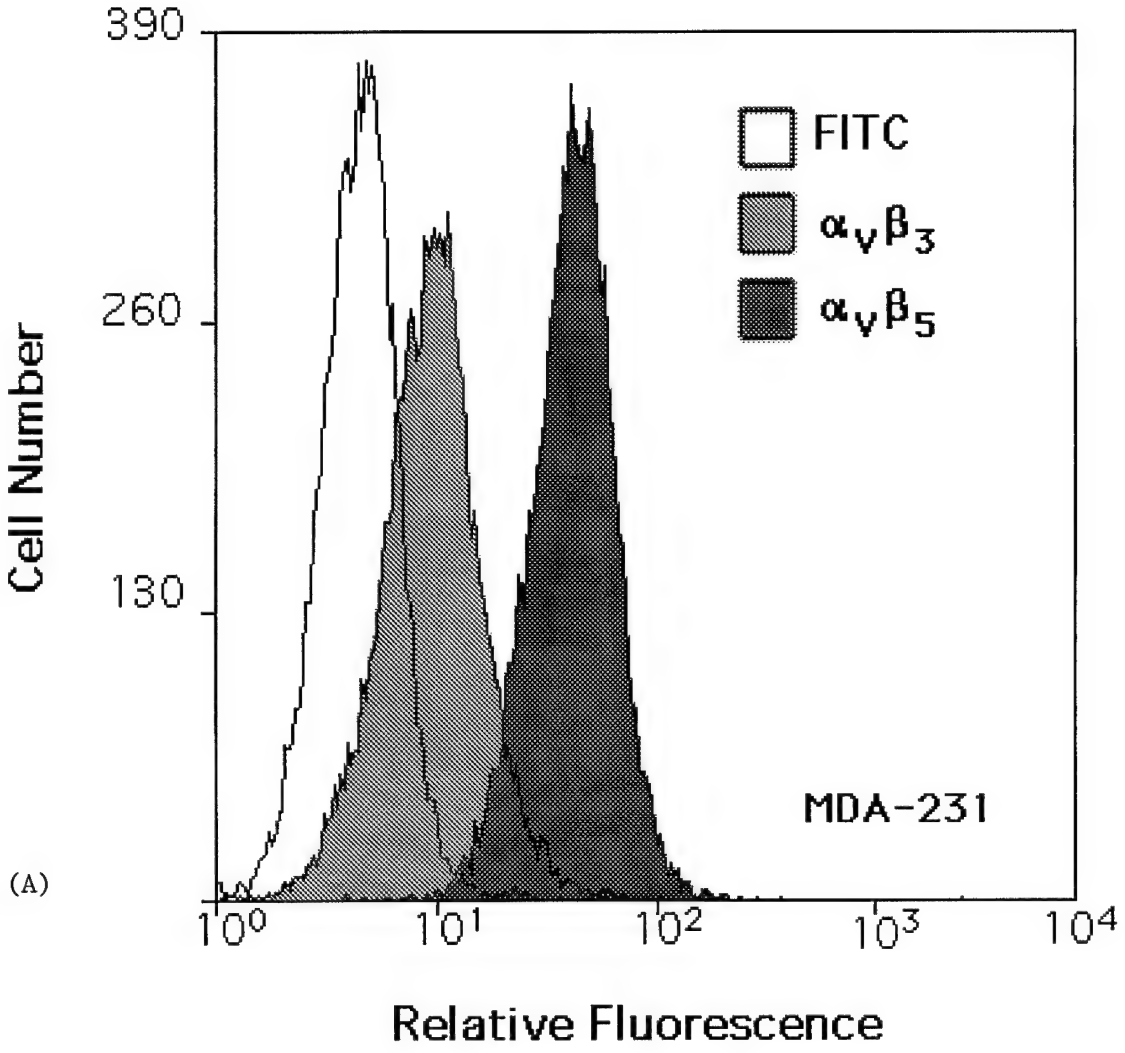
(B)


Attachment (O.D. 540 nm)
1.5
1
0.5
0
PL
VN
+ av
+avb5
BRB 10
+av
+avb3
+avb5
+av
+avb3
+avb5
BRB9
CBA4
BA3

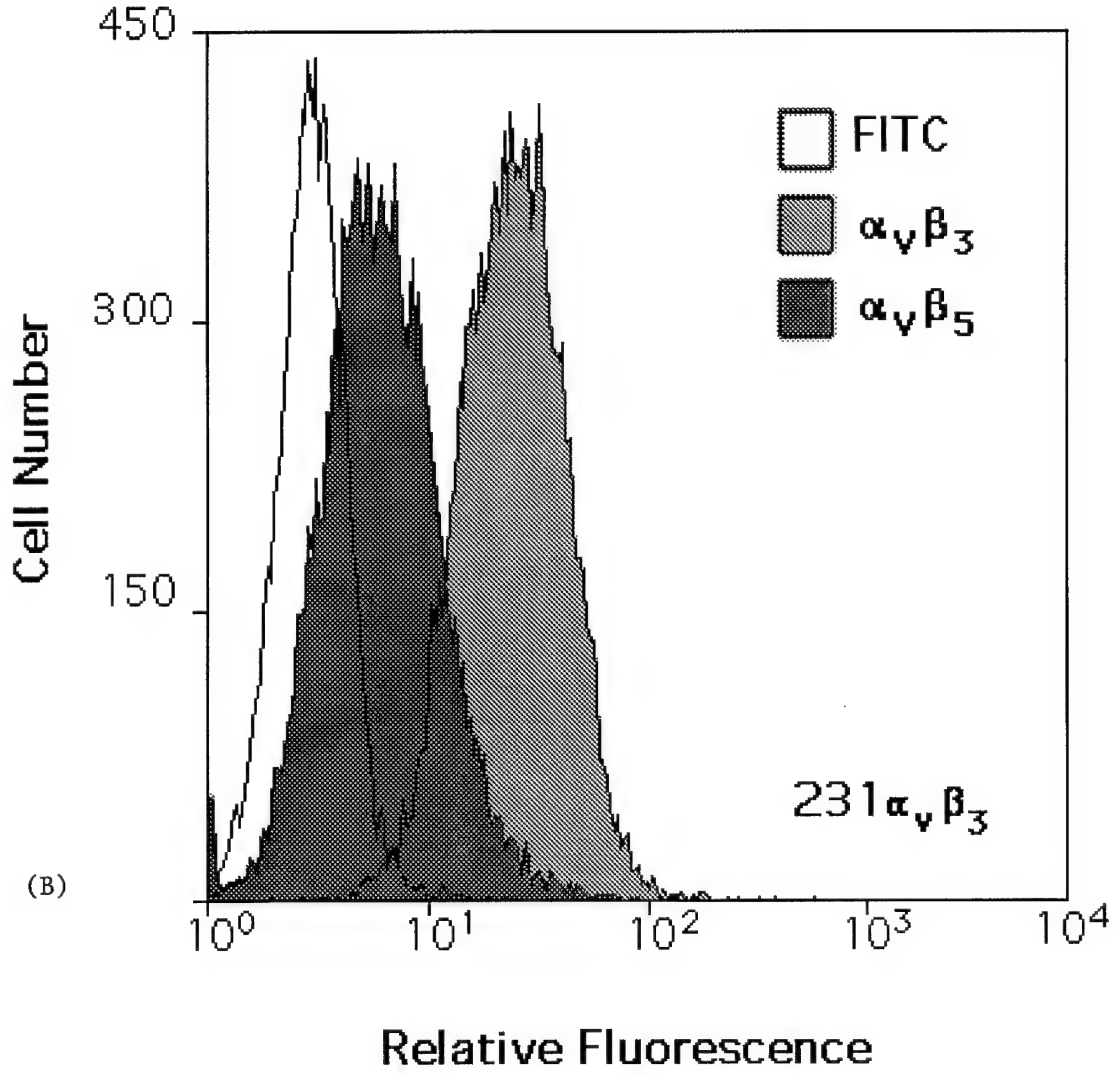
(C)

Figure 9

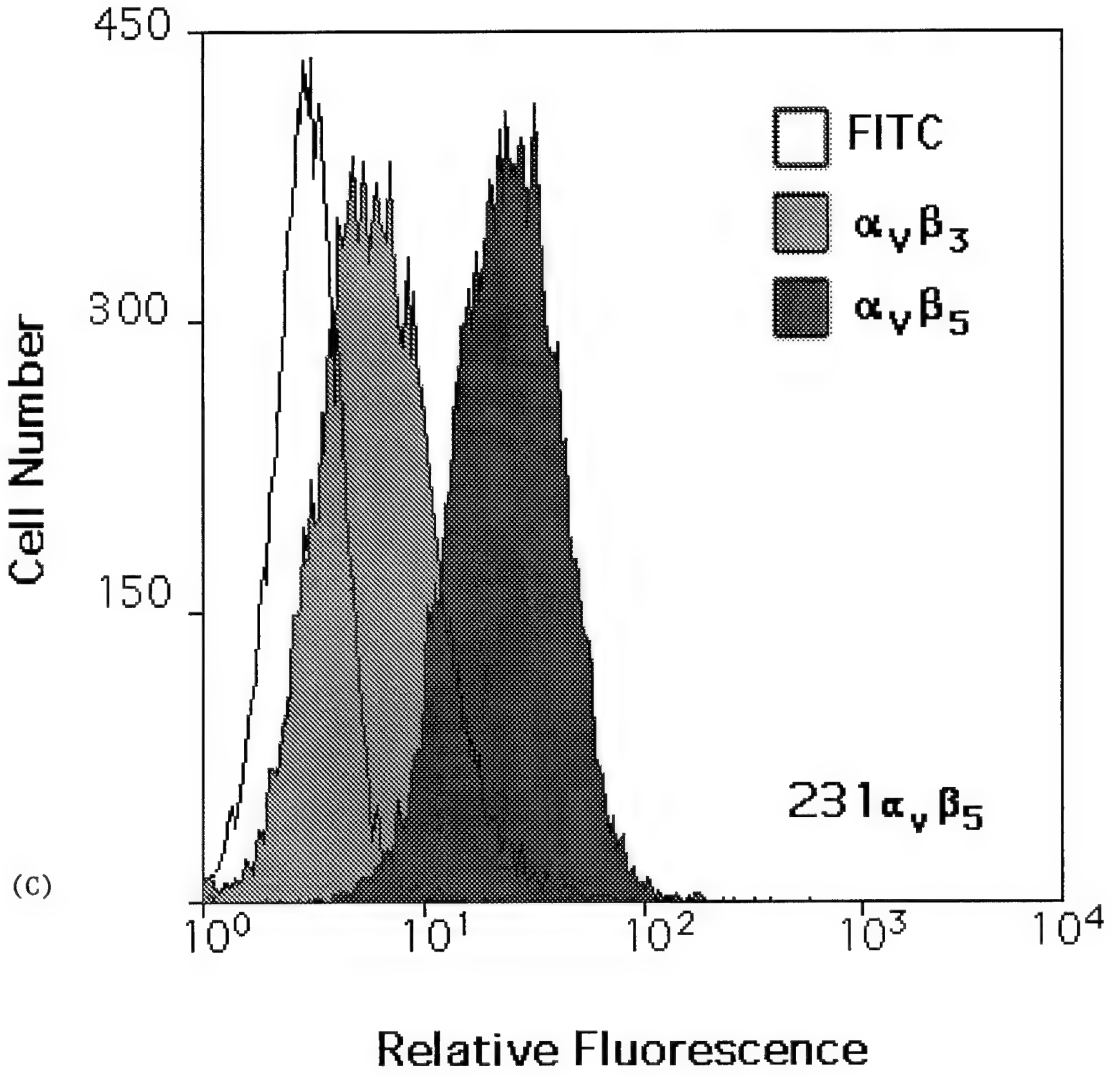
Integrin profiles of HBC cell lines MDA-231 **(A)**, MDA-231αvβ3 selectants **(B)** and MDA-231αvβ5 selectants **(C)** as analyzed by fluorescence activated cell sorting. FITC represents basal fluorescence (negative control).


390
260
130
Cell Number
FITC
$\alpha_V\beta_3$
$\alpha_V\beta_5$
MDA-231
10^0
10^1
10^2
10^3
10^4
Relative Fluorescence

(A)


450
300
150
Cell Number
FITC
$\alpha_v\beta_3$
$\alpha_v\beta_5$
231$\alpha_v\beta_3$
10^0
10^1
10^2
10^3
10^4
Relative Fluorescence

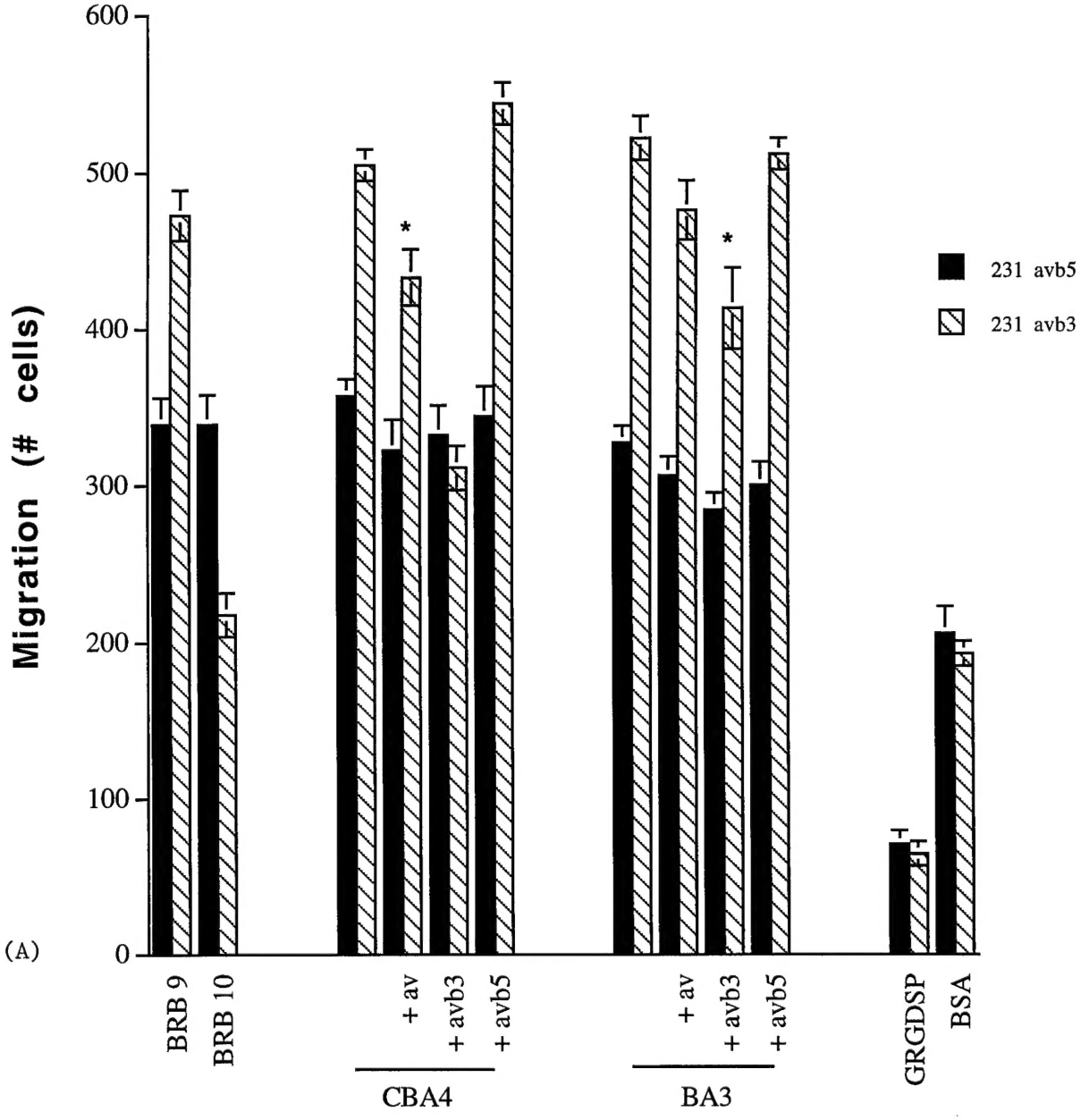
(B)

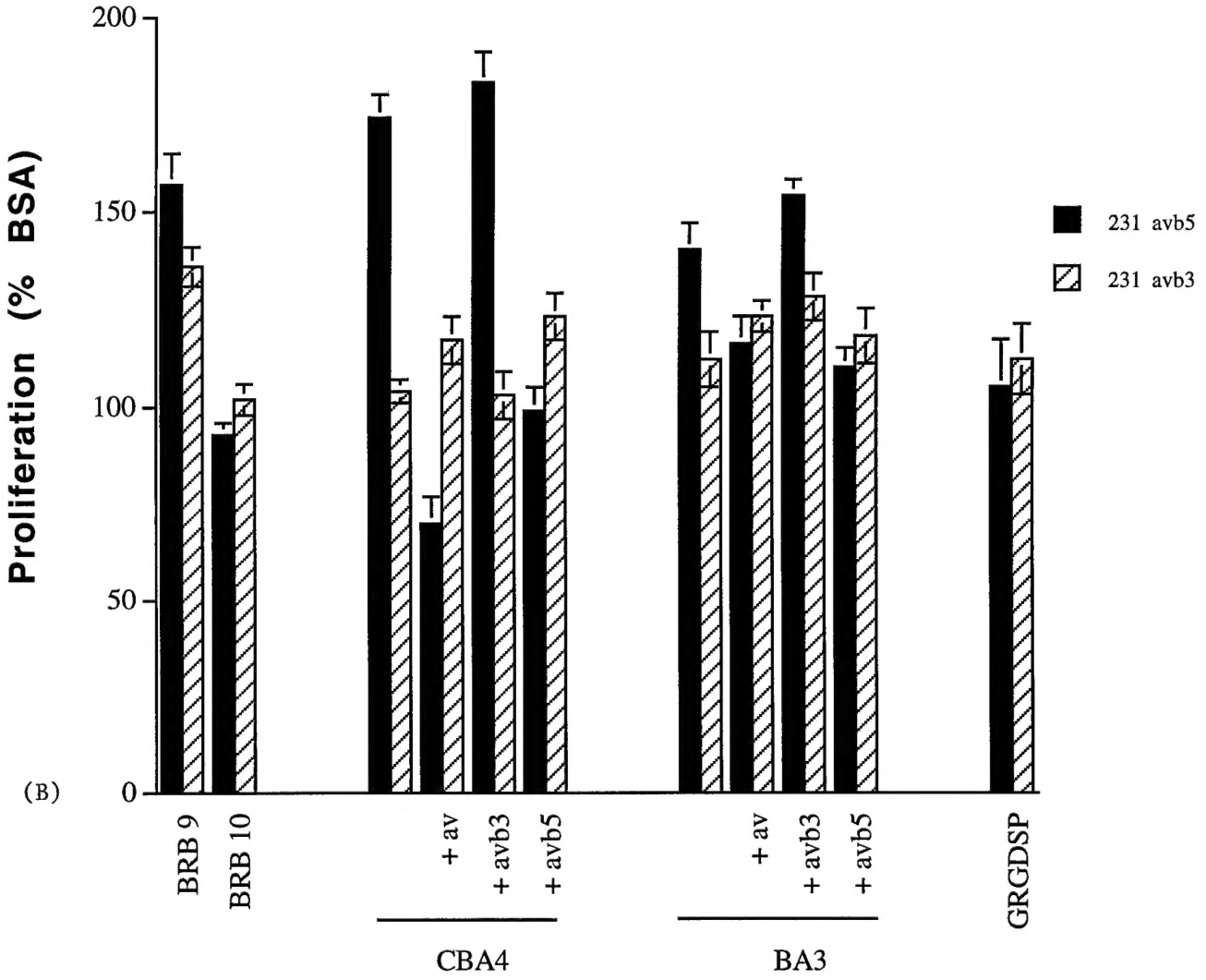

450
300
150
Cell Number
FITC
$\alpha_v\beta_3$
$\alpha_v\beta_5$
231$\alpha_v\beta_5$
10^0
10^1
10^2
10^3
10^4
Relative Fluorescence

(C)

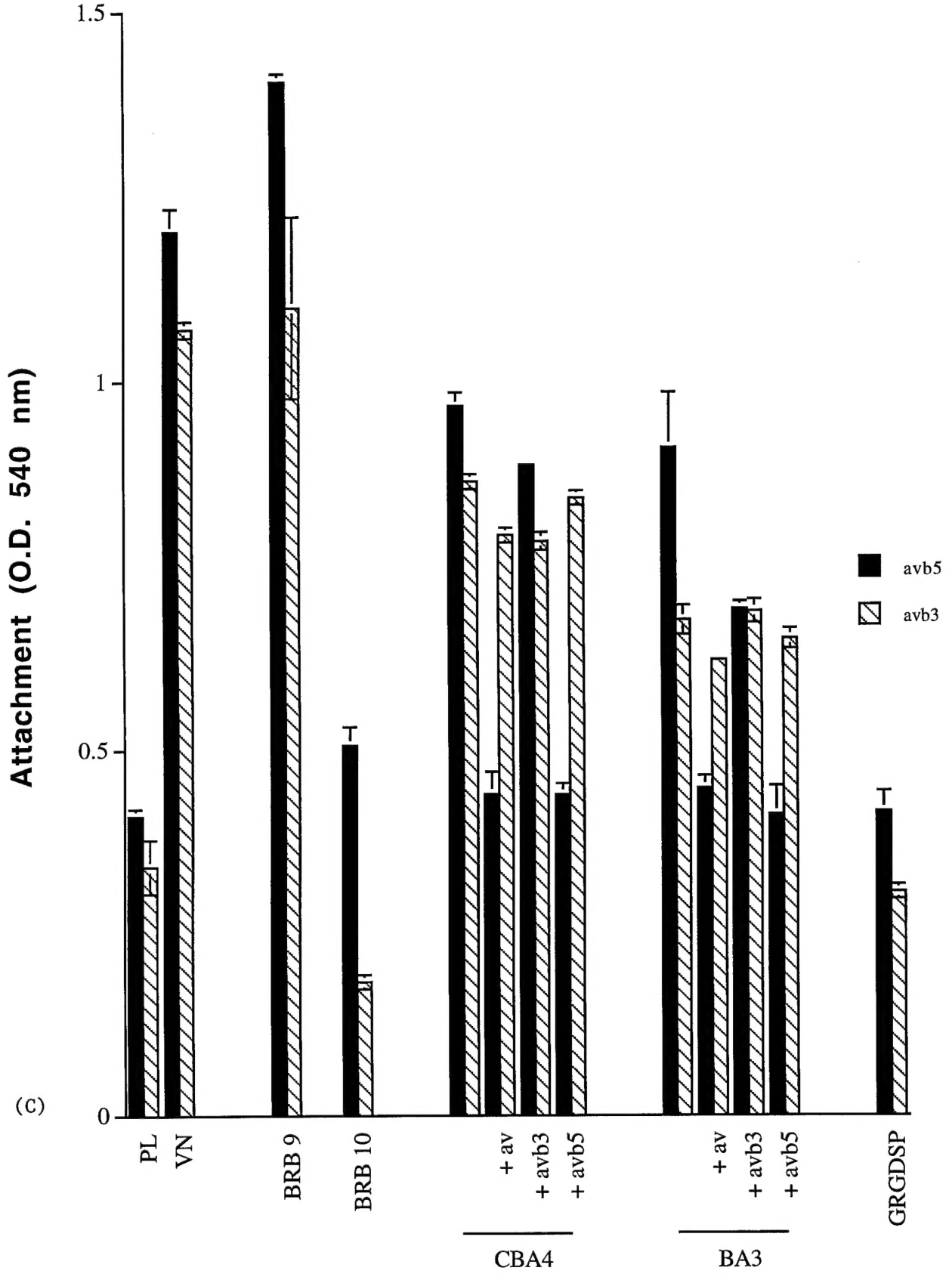
Figure 10

The effects of αv, αvβ3 and αvβ5 integrin blocking antibodies on MDA-231αvβ3 and MDA-231αvβ5 selected subpopulations for migration **(A)**, proliferation **(B),** and attachment **(C)** to BSP-derived RGD peptides CBA4 and BA3. Recombinant BSP fragments BRB9 and BRB10 are also shown as reference points. BSA (bovine serum albumin), GRGDSP (mutated fibronectin-derived RGD peptide), PL (plastic),and VN (vitronectin). Bars represent mean and standard deviation from triplicate filters/wells of a representative experiment. αv-blocked migration of 231αvβ3 selectants toward CBA4 is significantly lower than migration toward CBA4 alone, and αvβ3-blocked migration toward BA3 is significantly lower than toward BA3 alone. *$P<0.05$ in two-sided t-test.


Migration (# cells)
600
500
400
300
200
100
0
(A)
231 avb5
231 avb3
*
*
BRB 9
BRB 10
+ av
+ avb3
+ avb5
CBA4
+ av
+ avb3
+ avb5
BA3
GRGDSP
BSA


Proliferation (% BSA)
200
150
100
50
0
231 avb5
231 avb3
BRB 9
BRB 10
+ av
+ avb3
+ avb5
+ av
+ avb3
+ avb5
GRGDSP
CBA4
BA3

(B)


Attachment (O.D. 540 nm)
1.5
1
0.5
0
avb5
avb3
PL
VN
BRB 9
BRB 10
+ av
+ avb3
+ avb5
+ av
+ avb3
+ avb5
GRGDSP
CBA4
BA3

(C)